Development and validation of animal-free test methods to predict the skin sensitizing potential of chemicals

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[1] Natsch, A; Bauch, C.; Foertsch, L; Gerberick, F; Norman, K; Hilberer, A; Inglis, H; Landsiedel, R; Onken, S; Reuter, H; Schepky, A; Emter, R: *The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers in vitro: Results of a ring-study in five laboratories.* **Toxicology in vitro 25 (3)**, p 733-744 DOI: 10.1016/j.tiv.2010.12.014; 2011

[2] Bauch C.; Kolle S. N.; Fabian E.; Pachel C.; Ramirez, T.; Wiench B.; Wruck, C.J.; van Ravenzwaay, B; Landsiedel, R.: *Intralaboratory validation of four in vitro assays for the prediction of the skin sensitizing potential of chemicals*; **Toxicology in vitro 25 (6)** SI p. 1162-1168; DOI: 10.1016/j.tiv.2011.05.030; 2011

[3] Bauch C.; Kolle S. N.; Ramirez, T.; Eltze, T.; Fabian E.; Mehling, A.; Teubner, W.; van Ravenzwaay, B; Landsiedel, R.: *Putting the parts together: Combining in vitro methods to test for skin sensitizing potentials.*; **Regulatory Toxicology and Pharmacology 63 (3)**, p.489-504; DOI: 10.1016/j.yrtph.2012.05.013; 2012

[4] **Bauch C.**; Dearman R.J.; Kimber I., van Ravenzwaay B.; Landsiedel, R.: *Characterization of haptenization of chemical sensitizer on the cell surface*

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“It's one thing to feel that you are on the right path,

but it's another to think that yours is the only path.”

Paulo Coelho, *The Alchemist*

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1 ABSTRACT

1.1 SUMMARY

Skin sensitization is the development of the allergic contact dermatitis caused by chemicals. Regulatory accepted methods to assess skin sensitizing potential of chemicals are animal based tests, but increasing interest in animal welfare presses the development of animal-free methods. The aim of this work was the development, establishment and validation of several alternative methods to animal testing to predict the skin sensitizing potential of chemicals. Therefore several methods reflecting different parts of the complex sensitization process have been used. Three steps of the skin sensitizing process were depicted: protein reactivity of chemicals, activation of keratinocytes and dendritic cell like cells have been investigated. Establishment and validation of the methods was performed with 54 test substances of known sensitizing potential, and the findings were compared to available human patch test data and murine local lymph node assay data. The experimental data were used to calculate the predictivity of each assay in order to compare the several assays and as well to evaluate possible combinations. By combination of different assays or more specifically the combination of the outcome of different assay into a testing strategy or testing battery predictivities were increased and allowed the prediction of sensitizing and non-sensitizing substances with high probability.

The development of new methods to predict the protein reactive potential of sensitizing substances indicated that such compounds are able to react with proteins on the cell surface and that this reaction impacts the detection of such proteins by antibody staining. After treatment with sensitizing substances less protein was detectable and two hypotheses were proposed and investigated: It was shown that antibody binding was reduced or fully inhibited but also that the internalization of the altered protein was triggered. A correlation between the sensitizing potential and the altered protein level was confirmed although the underlying mechanism of how detectable protein level were reduced remained to be not fully understood. Further investigations are still required as the expression of the used protein in this study is up-regulated during the activation of dendritic cells. This phenomenon was observed for three out of five sensitizing substances.

This work represents successful validations, inter- and intra-laboraty, of several animal-free test methods. The combinations of assays showed that predictivity compared to single assays can be increased and that for the number of tested substances these combinations are comparable to the local lymph node assay, the current gold standard to assess skin sensitizing potential. Although this work was no part of the formal validation of these assays, increased acceptance was gained, showing the reliability, reproducibility and high predictivities of these assays. The work on a new cell based protein reactivity assay showed a correlation between reduced level of detectable protein and the exposure of cells with sensitizers, although more investigation is required. The expression of the chosen protein unfortunately seemed to be up-regulated under the presence of skin sensitizer. Investigation of proteins their expression is independent of cell activation may provide more stable results.

1.2 ZUSAMMENFASSUNG

Die Entstehung der Allergischen Kontaktdermatitis ist besser bekannt als Hautsensibilisierung welche durch Chemikalien ausgelöst wird. Um das sensibilisierende Potential der Chemikalien zu untersuchen sind zur Zeit nur an Tieren durchgeführte Methoden regulatorisch zugelassen. Mit wachsendem Tierschutzinteresse wird die Entwicklung neuer, versuchtierfreier Methoden gefordert. Das Ziel dieser Arbeit war die Entwicklung, Etablierung und Validierung verschiedener Methoden, welche als Ersatz zu Tierversuchsmodellen zur Vorhersage eines hautsensibilisierenden Potentials von Chemikalien eingesetzt werden sollen. Hierfür wurden drei verschiedene Teilschritte des komplexen Prozesses der Hautsensibilisierung in verschiedenen Methoden abgebildet. Dabei wurden die Proteinreaktivität von Chemikalien und die Aktivierung von Keratinozyten sowie von Dendritschen Zellen untersucht. Die Etablierung und Validierung wurden anhand einer Auswahl von insgesamt 54 Substanzen durchgeführt und die Ergebnisse mit verfügbaren humanen Patch-Test Daten bzw. Daten aus dem Maustest, lokaler Lymphknotetest, verglichen. Mit diesem Vergleich konnten die Prädiktivitäten der einzelnen Methoden berechnet werden, so dass die Methoden untereinander verglichen und sinnvolle Kombinationen identifiziert werden können. Die Kombinationen einzelner Methoden bzw. der Ergebnisse der Methoden zu einer Teststrategie oder -batterie erhöhte die Prädiktivitäten im Vergleich zu einzelnen Methoden und ermöglichte es, eine Vorhersage von sensibilisierenden bzw. nicht-sensibilisierenden Substanzen mit hohen Wahrscheinlichkeiten zu treffen.

Die Entwicklung einer neuer Methoden zur Vorhersage des proteinreaktiven Potentials zeigte, das sensibilisierenden Substanzen mit Proteinen an der Zelloberfläche reagieren und Effekte auf die Detektion von Zelloberflächenmolekülen mittels Antikörperfärbung haben können. Nach Behandlung der Zellen mit sensibilisierenden Substanzen konnte weniger Protein an der Zelloberfläche festgestellt werden. Hierzu wurden zwei unterschiedliche Hypothesen aufgestellt und experimentell überprüft. Es wurde gezeigt, dass die Reaktion von Chemikalien mit Proteinen an der Zelloberfläche die Bindung von Antikörpern reduziert oder vollständig inhibiert, aber auch die Internalisierung des veränderten Proteins auslöst. Ein Zusammenhang zwischen der sensibilisierenden Eigenschaft von Chemikalien und der reduzierten nachweisbaren Proteinmenge wurde gezeigt, obwohl der Mechanismus welcher der Reduktion des nachweisbaren Proteins zu Grunde liegt nicht vollständig geklärt werden konnte. Nicht sensibilisierende Substanzen hatten keinen Effekt auf den nachweisbaren Proteingehalt. Weitere Untersuchungen sind nötig, da die Expression des untersuchten Proteins mit Aktivierung dendritischer Zellen hochreguliert werden kann. Diese Phänomen wurde bei drei von fünf sensibilisierenden Substanzen beobachtet.

Diese Arbeit zeigte die erfolgreiche Validierungen verschiedener versuchstierfreier Methoden, welche zum einen intern aber auch teilweise mit externen Laboren als Ringstudien durch geführt wurde. Es hat sich gezeigt, dass die Kombination dieser Methoden die Aussagekraft im Vergleich zu den einzelnen Methoden erhöht hat und, für die Anzahl der getesteten Substanzen, vergleichbare Werte zum Standardtest, dem Lokalen Lymphknoten Test, erzielt wurden. Obwohl diese Ringversuche nicht Teil der offiziellen Validierung der einzelnen Tests waren, wurde die Anerkennung durch die gezeigte Zuverlässigkeit, Reproduzierbarkeit und

hohe Prädiktivitäten erhöht. Die Entwicklung eines neuen zellbasierten Tests zur Untersuchung der Proteinreaktivität zeigte, dass es einen Zusammenhang zwischen der erniedrigten, messbaren Menge des Proteins und der Behandlung der Zellen mit Sensibilisierer gibt, dennoch sind weitere Experimente nötig. Die Expression des gewählten Proteins wurde teilweise durch die Behandlung mit Sensibilisierer hoch reguliert. Proteine, deren Expression unabhängig von aktivierten Zellen ist, ergeben möglicherweise stabilere Ergebnisse.

2 INTRODUCTION

The use of animal-free methods to predict the hazard and risk of chemicals, such as their skin sensitizing potential, is becoming more and more important due to public interest in animal welfare and the demands by legal bodies. The scientific knowledge of underlying cellular processes and responses allows the description, development and establishment of new methods. Before a newly developed method can be used for regulatory purposes the validity and accuracy has to be proven, which are time consuming processes. In case of skin sensitization several cellular processes are involved which are unlikely to be sufficiently reflected in a single alternative method. The process of skin sensitization can be reflected using cell based assays, but also computer based structural analyses of chemicals and cell-free approaches. The challenge of development of animal-free test methods is not only the establishment of several assays to rebuild the process of sensitization *in vitro* but also to obtain reliable analyses and judgment of the sensitizing potential of chemicals.

2.1 ALLERGIC CONTACT DERMATITIS AND CHEMICAL ALLERGENS

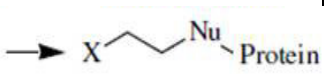
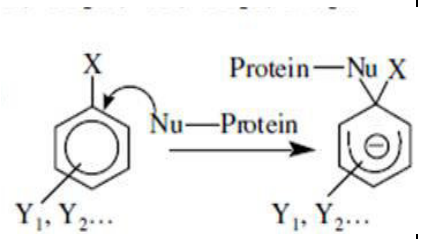
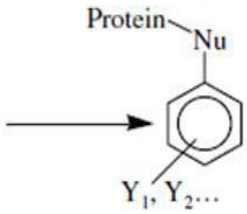
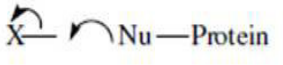
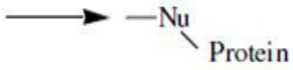
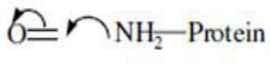
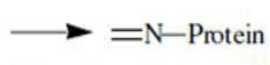
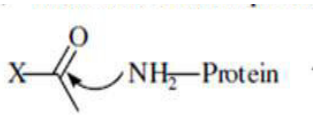
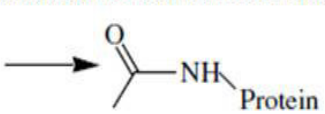
Chemicals can be the trigger of allergic contact dermatitis (ACD), also known as contact allergy. The characteristic skin condition is noticeable with symptoms such as rash or skin lesions, itchiness, ooze or drain and appearance of blisters and desquamation or redness of the skin. ACD and occurring symptoms can range from light to severe reactions (Hostynek and Maybach 2004; Pezutto *et al.* 2006; Rustemeyer *et al.* 2006). ACD is a type IV allergic reaction of the skin mediated by T cells and triggered by industrial chemicals, cosmetics or hygienic products or one of their ingredients (Coombs and Gell 1975; Krasteva *et al.* 1999). ACD is a two-phase-process, consisting of development and elicitation. The development of ACD is also called skin sensitization and occurs upon first contact with the allergen and proceeds without any noticeable symptoms (Saint-Mezard *et al.* 2004). The elicitation, the actual clinical manifestation of ACD, occurs after the completed sensitization phase and with repeated contact to the allergen (Saint-Mezard *et al.* 2004). Once sensitized to a chemical, ACD will last for a lifetime. Today, there is no cure for ACD, however, important aspects in the treatment of this condition and prevention of the outbreak are to identify and to avoid the allergen (Peiser *et al.* 2012; Schnuch *et al.* 2008; Uter *et al.* 2006). ACD entails high impact on work and social life (Hutchings *et al.* 2001), hence there is a high interest by government and industry for safe handling of raw materials by workers and risk-free use of finished products for consumers (Kimber *et al.* 2002a; Smith-Pease 2003). Nowadays about 15 – 20 % of the population suffer from ACD, mainly people in the Western world, with an increasing tendency (Thyssen *et al.* 2007; Zug *et al.* 2009). Risk factors for the development of ACD are sex, age and genetic predisposition, where the repeated contact with low molecular weight allergens at the workplace or with the use of consumer products is the main trigger (Ngyen *et al.* 2008; Peiser *et al.* 2012; Smith-Pease 2003). Prominent examples for skin sensitizing chemicals are fragrances like and dyes in cosmetics and nickel in jewelry (Kimber *et al.* 2002a; Thyssen *et al.* 2007).

2.1.1 CHEMICAL ALLERGENS

Most chemical allergens are of low molecular weight (below 500 g/mol) and electrophilic molecules, so-called haptens. Due to their small molecular structure haptens are not immunogenic. This implies that the immune system fails to recognize the haptens without a stable conjugation to proteins, also known as haptenization. So-called carrier proteins enables haptens to trigger the immune response. Till now researchers were not able to identify or describe a specific protein or protein family as carrier proteins for chemical allergens, but nevertheless it was shown that proteins with nucleophilic moieties of amino acids like lysine, histidine and cysteine serve as main reaction partners (Ahlfors *et al.* 2003; Divkovic *et al.* 2005; Dupuis and Benezra 1982; Landsteiner and Jacobs 1936; Lepoittevin *et al.* 1998; Smith and Hotchkiss 2001; Weltzien *et al.* 1996). Thus the event of haptenization can occur with any protein in the skin (Rock and Goldberg 1999; Smith-Pease 2003). With the inherent electrophilic property of haptens they are able to react with the nucleophilic residues of cysteine, lysine or histidne (Roberts and Lepoittevin 1998), although some others, known as pre- and pro-haptens, require previous activation (Dupuis and Benezra 1982; Smith and Hotchkiss 2001). Pre-haptens can acquire protein reactivity by auotoxidation (Hagvall *et al.* 2007; Lepoittevin 2006). For instance, a prominent pre-hapten is *para*-phenylenediamine, used in hair dye products e.g. as ingredient of the so-called black henna (Ho *et al.* 2004; McFadden *et al.* 2007). Pro-haptens can be converted into reactive molecules by skin enzymes such as cyotchrome P450 enzymes or flavin-dependent monooxygenases (Bergstrom *et al.* 2007; Hagvall *et al.* 2007; Karlberg *et al.* 2007; Smith and Hotchkiss 2001). Some representatitves of pro-haptens are cinnamic alcohol, geraniol and 4 allyl-anisol, all used as fragrances in cosmetics (Jaeckh *et al.* 2012; Schnuch *et al.* 2004; Schnuch *et al.* 2007).

Haptens form a large spectrum of different substance classes such as aldehydes, anhydrides, thiazoles, ethers, esters, and metal salts and many others. A classification of sensitizers according their electrophilic reactivity mechanism was suggested (Roberts *et al.* 2007c). Roberts and co-workers classified a range of known haptens according to their reaction mechanism shown in Table 1. Chemical sensitizers are able to react with nucleophilic molecules via Michael reaction, nucleophilic (aromatic) substitutions (SN2 or SNAR), Schiff base reactions and acetylation. Metal allergens such as nickel and copper form coordination bonds instead of covalent bonds. Such complexations of metals occur e.g. with histidine rich proteins (Thierse *et al.* 2004). It was shown that sensitizing potential of chemicals can be predicted using their reactivity scheme (Patlewicz *et al.* 2008).

Table 1: Reaction mechanism involved in skin sensitization
The table shows simplified reaction mechanism for Michael acceptors, SNAr electrophile, SN2 electrophiles, Schiff base formers and acylating agents with protein nucelophiles (Aptula et al. 2006)

Mechanistic domain	**Protein binding reaction**	**Modified protein**	**Identification characteristics**
Michael acceptors			Double or triple bond with electron withdrawing substituent X such as –CHO, -COR, -CO_2R, -CN, -SO_2R, -NO_2
SNAr electrophiles			X = (pseudo) halogen, Y's = electron withdrawing groups like -NO_2, -CN, -CHO
SN2 electrophiles			X = halogen or leaving group bonded to primary alkyl, benzylic or allylic carbon
Schiff' base formers			Reactive carbonyl compounds such as aliphatic aldehydes, some α,β or α,γ diketones, -ketoester
Acylating agents			X = halogen or leaving group (-OC_6H_5 such that XH is acidic enough for X- to act as good leaving group

2.1.2 PHASES OF ACD: SKIN SENSITIZATION AND ELICITATION

ACD can be described in the two phases: 1) sensitization phase, corresponding to the symptom-free development of ACD, and 2) elicitation phase, which is the symptomatic appearance of ACD (Saint-Mezard *et al.* 2004). The adverse outcome pathway (AOP), key events and consecutive processes involved in the mechanism and mode of toxic action, of skin sensitization is complex and involves different cell types, cellular responses and vice versa results in activation of cells in the skin (Fig. 1) (OECD 2012). The skin is the largest organ and represent a barrier to the environment (Janeway; Efferth; Marquardt, Meyer *et al.*, 2007). Three layers of different tissue build the skin, namely: epidermis, dermis and cutis, whereas the epidermis is the import part

of the skin in the sensitization process. The epidermis represents the outer layer of the skin and functions as protector against harmful environmental effects, microorganism and other influences but also it has an important role in immunology (Meyer *et al*., 2007). Keratinocytes (KC) are the main cell type in the epidermis with a frequency of 95%, next to Merkel cells, melanocytes and Langerhans' cells (LC), the latter are dendritic cells (Marquardt). If a hapten penetrates through the *stratum corneum*, the outer layer of the epidermis and main physical penetration barrier, it reaches the layer within the epidermis where it reacts with proteins to form stable conjugates, the complete allergen (Dupuis and Benezra 1982; Landsteiner and Jacobs 1936; Weltzien *et al.* 1996). If the hapten is not inherently reactive it can be converted to reactive species by autoxidation or enzymatic conversion of enzymes mainly from KC (Bergstrom *et al.* 2007; Svensson 2008). KC can be activated by the presence of hapten-protein-conjugates. Activated KC release danger signals in form of cytokines like interleukin (IL)-1α and tumor necrosis factor α (TNFα) and facilitates the activation of immature dendritic cells (iDC) and hence the inflammatory response. (Kim *et al.* 2009; Kimber and Cumberbatch 1992). iDC are antigen presenting cells (APC) of the skin and more specifically called LC if found in the epidermis. Upon recognition and up-take of hapten-protein conjugates iDCs become activated (Aiba *et al.* 1997). Whilst DC undergo a process of maturation, the hapten-protein conjugate is degraded and its fragments are presented on the cell surfaces via major histocompatibility complex (MHC) class I and II (Macatonia *et al.* 1987). Down-regulation of adhesion molecules such as E-cadherin allow DCs to detach from surrounding tissue (Schwarzenberger and Udey 1996). By means of caspase-1 (Antonopoulos *et al.* 2001) and matrix metalloproteinases to penetrate cell-cell contacts of the tissue (Ratzinger *et al.* 2002), an interplay of cytokines (Antonopoulos *et al.* 2008; Cumberbatch *et al.* 2001) and the simultaneous down-regulation of C-C chemokine receptor type (CCR)2 for skin homing and up-regulation of CCR7 for lymph node homing, DCs migrate out of the skin towards the local lymph node where they encounter naïve T cells (Ohl *et al.* 2004; Sallusto *et al.* 1998). Mature DCs carry cell surface proteins such as CD40, CD80 and CD86, all three so-called maturation markers, to facilitate the cell-cell interactions with T cells in addition to antigen-MHC molecules (Kimber and Cumberbatch 1992; Hulette *et al*. 2002; Steinman 2001; Weltzien *et al.* 1996). If a T cell carries the antigen matching T cell receptor (TCR) its activation, differentiation and proliferation into antigen specific T cells takes place (Banchereau and Steinman 1998; Weltzien *et al.* 1996). Development of skin sensitization may require several days, from first contact to T cell activation. (Banchereau *et al.* 2000; Kimber and Cumberbatch 1992; Roychowdhury and Svensson 2005; Ryan *et al.* 2007; Toebak *et al.* 2009)

Upon completion of the sensitization phase, specific T cells circulate in the blood stream until the following allergen contact. In the event of such a subsequent contact with the allergen the process is similar to the sensitization with haptenization, stimulation of KCs to release cytokines, and activation of DCs. Specific T cells infiltrate the site of allergen contact and interaction of DC and T cells triggers elicitation of ACD with the characteristic symptoms of rash or skin lesions, itchiness, ooze or drain and appearance of blisters, desquamation or redness of the skin appear caused by proliferation of KC, vasodilatation and edema (Banchereau and Steinman 1998; Pezutto *et al.* 2006).

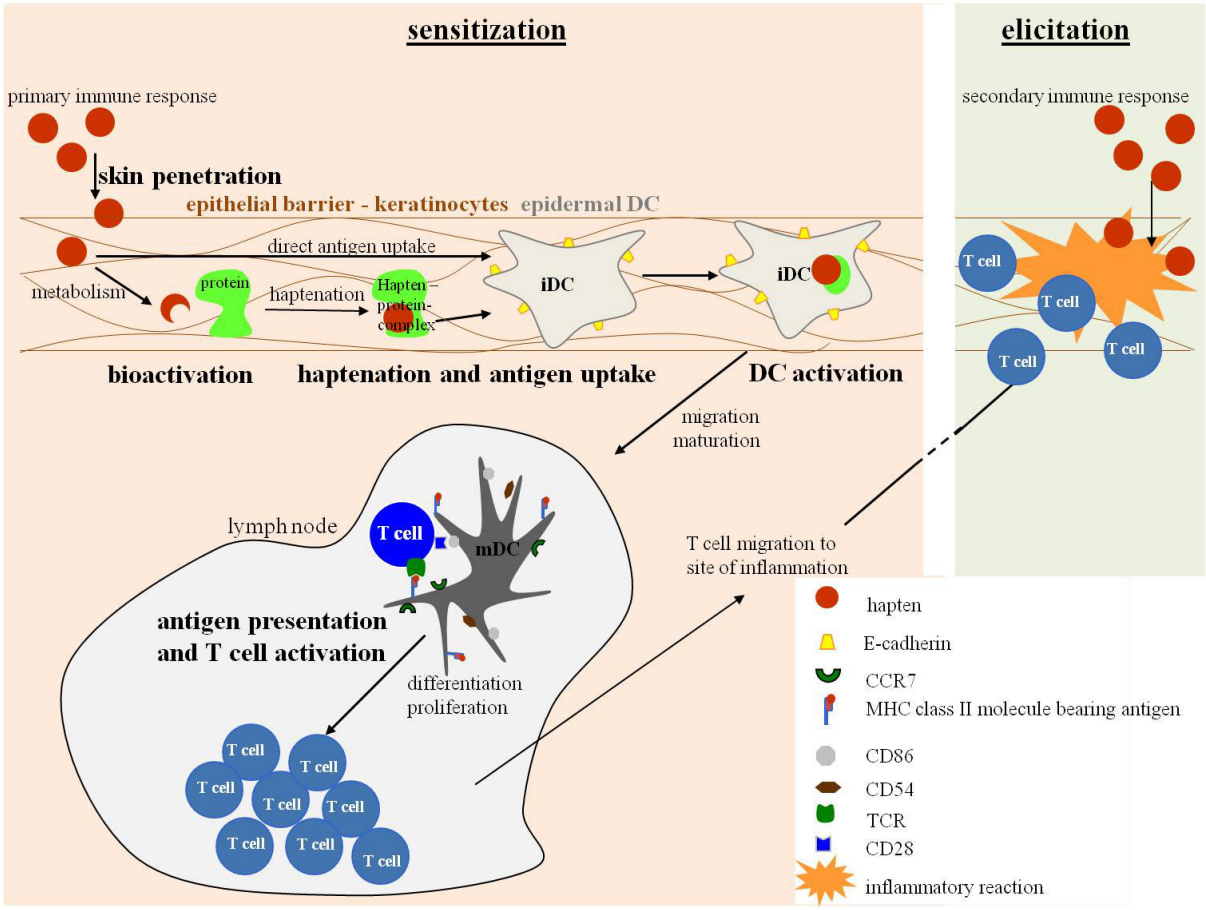


Figure 1: Process of sensitization and elicitation
Skin sensitization includes the steps of (1) penetration of the hapten through the skin, (2) activation of not inherently reactive haptens by enzymes, (3) protein reaction and uptake of the hapten-protein conjugate, (4) DC activation with maturation and migration of DC to the local lymph node, and finally (5) antigen presentation to and activation of naïve T cells. Elicitation occurs after subsequent contact with the allergen and the entailed (6) infiltration of specific T cells into site of allergen contact triggering the inflammatory reaction. Modified according Banchereau *et al.* 2000, Kimber and Cumberbatch 1992, Roychowdhury *et al.* 2005 and Ryan *et al.* 2007, Bauch 2009.

2.2 IDENTIFICATION OF SKIN SENSITIZERS

2.2.1 LEGAL BACKGROUND IN SKIN SENSITIZATION

The regulatory bodies, such as US Environmental Protection Agency, US Food and Drug Administration, European Chemical Agency or Organization for Economic Co-operation and Development (OECD), demand hazard and hazard potency assessment with the current standard methods namely the guinea pig maximization test (GPMT) and the murine local lymph node assay (LLNA). Both assays are implemented in OECD testing guidelines (OECD TG 406 1992; OECD TG 429 2010) issued by the OECD. There is an increasing interest in animal welfare and a demand by public and legal bodies to replace animal testing in general, and especially to replace LLNA and GPMT as skin sensitization tests with animal-free test methods. This is also stimulated by 7th amendment (2003/15/EC 2003) of the Cosmetic Directive (76/768/EEC 1976), now Cosmetic Regulation (EU 1223/2009 2009) and the regulation for the registration, evaluation, authorization and restriction of chemicals (REACH), a regulation by the European Union. Hence, over the last years, animal-free testing methods for

industrial chemicals, pharmaceuticals, cosmetics and agrochemicals became a high priority. The European Commission banned the use of animal experiments in 1986 only if scientifically approved alternatives were available (European Commisson 1986), whereas the 7th amendment of the Cosmetic Directive implemented a testing ban for cosmetic products and ingredients on animals and the marketing of such animal-tested products in 2009, except of some the endpoints for repeated-dose toxicity, reproductive toxicity and toxicokinetics coming into force in 2013, like skin sensitization.

2.2.2 STANDARD SKIN SENSITIZATION TEST METHODS

2.2.2.1 GUINEA PIG TESTS

So far, the skin sensitizing potential of industrial chemicals, cosmetic ingredients, and agrochemicals has been identified through animal testing. Guinea pigs and mice serve as standard animal models and both methods were outlined testing guidelines published by the OECD. The protocols of the guinea pigs were set in the OECD guideline no. 406 in 1981 and was revised in 1992 (OECD TG 406 1992). The guinea pigs can be treated in two different ways. The substance is dissolved in Freund's complete adjuvant/water or physiological saline and is either intradermal injected or applied on the skin, followed by a resting phase of 10 to 14 days to allow the development of the immune response. The highest dose should only cause a mild to moderate skin irritation. The animals are challenged with another, non-irritating dose, and the extent and degree of the skin reaction is assessed in comparison to control animals. The test requires 10 to 20 animals per dosage and 5 to 10 animals per control group (OECD TG 406 1992). Two varieties of the guinea pig test are in use which differ in the use of adjuvant and the duration of the assay. The maximization test (GPMT) by Magnusson and Kligman is performed using Freund's complete adjuvant, mineral oil solution, and needs 24 days in total (Magnusson and Kligman 1969). The second version is the so-called Buehler test (Buehler 1965), a non-adjuvant guinea pig test. Assay duration is 32 days. The guinea pig tests cover of sensitization and elicitation phase, but the results rely on subjective readout skin reactions. And the limitation to hazard potential only (binary (yes/no) answer) with a lack of potency information (Mehling *et al.* 2012; OECD TG 406 1992).

2.2.2.2 LOCAL LYMPH NODE ASSAY

The LLNA procedure, outlined in the OCED guideline 429 in 2002 and revised in 2010 (OECD TG 429 2010) was developed by Kimber and co-workers (Kimber *et al.* 1989). For substance treatment chemicals are dissolved in solvents like acetone:olive oil (4:1), *N*,*N*-dimethylformamide, propylene glycol, methyl ethyl ketone or dimethyl sulfoxide. The maximum applied dose should be 100 % of liquid substances and maximum soluble concentrations for solid substances. Substance treatment is carried out by topical application on the dorsum of each ear per mouse and treatment group on three consecutive days (see Fig. 2). After a resting phase of two days mice are injected with ^{3}H-methyl thymidine and the local lymph nodes of each ear are excised after additional 5 hours of incubation. The lymph node cells are isolated and the ^{3}H decay from labeled thymidine is evaluated and indicates cell proliferation of lymph node cells. Stimulation indices (SI) are calculated and compared to control animals. SI of >3 indicate a test substance as skin sensitizer. According to the 3R concenpt (see chapter 2.3) the LLNA represents an alternative method to the GPMT, although it is still an *in vivo* assay

(OECD TG 429 2010). However, fewer number of animals are required (a total of 20 to 25 mice) and less harm is caused to animals compared to the GPMT, without a challenge phase and adjuvant. With the quantitative assessment of the cellular response of the immune system the LLNA results in objective and quantifiable readouts. Compared to human data, the LLNA provides some false positive predictions of substances and this has been discussed as one of its disadvantages (Basketter et al. 2009; Garcia et al. 2010; Kreiling et al. 2008; Penninks 2006) and the use of radioactive thymidine and consequent increased safety precautions are additional disadvantages. Since 2003 the LLNA is used for the assessment of skin sensitizing potency (Kimber *et al.* 2003). According to the test substance concentration which induces an SI >3 the substance is classified as extreme (<0.1 %), strong (from ≥0.1 % to <1 %), moderate (from ≥1 % to <10 %) and weak (from ≥10 % to <100 %). The LLNA was further optimized to reduce the number of animals and is implemented as the so-called reduced LLNA in the LLNA testing guideline (OECD TG 429 2010). Up to 40 % less animals are used compared to the traditional LLNA but forfeit the advantage of potency prediction as with less dose groups no dose-response can be assessed (Anderson 2011; OECD TG 429 2010). A non-radioactive protocol was published by replacing the ^{3}H-methyl thymidine with 5-bromo-2-deoxyuridine (BrdU) and a colorimetric readout of BrdU after cell lysation (Anderson 2011; Kolle *et al.* 2012a; Kolle *et al.* 2012b; OECD TG 429 2010). A list of reference substances to approve sufficient accuracy and reliability according the specified test purposes was described (Casati 2007) and a selection of 22 chemicals was published by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, USA) (Kolle *et al.* 2012b).

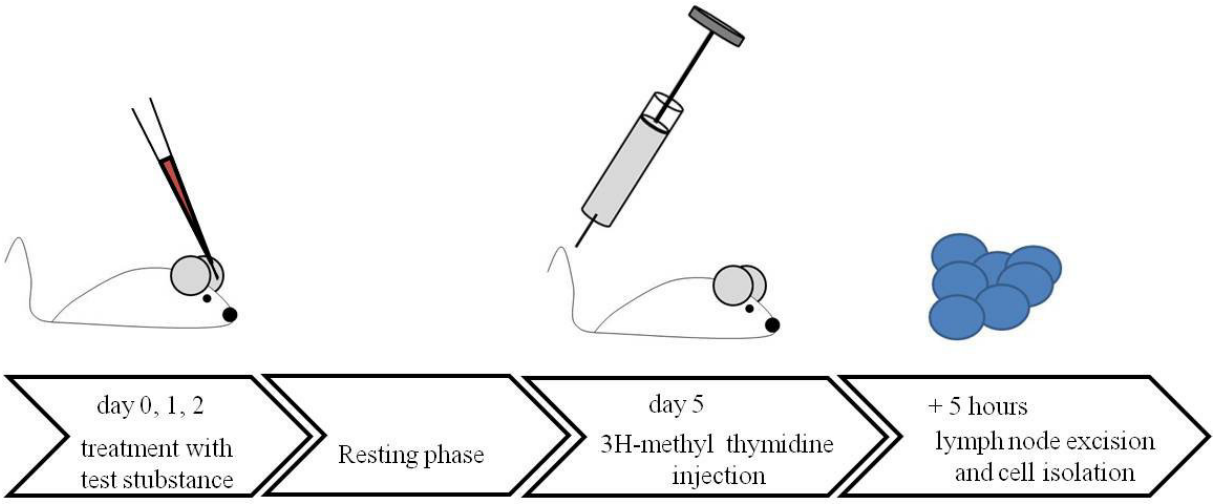


Figure 2: Procedure of LLNA (adopted from (OECD TG 429 2010))
Mice are treated on the dorsum of the ears with test substance on three consecutive days. After a resting phase of two days, mice are injected with ^{3}H-methyl thymidine. After five hours local lymph nodes of the ears are excised and ^{3}H decay is assessed to evaluate cell proliferation.

2.3 ALTERNATIVE METHODS TO PREDICT SKIN SENSITIZING POTENTIAL

A guiding principle for alternative methods was described in 1959 by the scientists Russel and Burch, in which they described the Reduction, Refinement and Replacement (3R) of animals and animal based test methods, known as the 3R concept (Russell and Burch 1959). As mentioned above there is high interest by public and regulatory bodies in replacing the LLNA and GPMT with alternative, animal-free test methods (2003/15/EC 2003; Mehling *et al.* 2012). Currently, for skin sensitization there is no regulatory acceptance of an animal-free method such as an available OECD test guideline or an endorsement by European Center for Validation of Alternative Methods (ECVAM, Italy), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, USA), the National Center for the Replacement, Refinement and Reduction of Animals in Research (NC3R, United Kingdom), Netherlands Centre for Alternatives to Animal Use (NCA, Netherlands) and the Centre for the Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET, Germany),

The development of assays to replace the current *in vivo* sensitization tests is a challenge due to the biological complexity of cellular processes involved in skin sensitization. To be able to assess skin sensitization *in vitro* the process has to be broken down into single steps and assessed by several methods and adequate and useful models, especially to simulate cell response, have to be identified. In case of cells it needs to be considered that they are fully functional in their physiological, cellular environment (Matzinger 2007; Matzinger and Kamala 2011) and that isolation of cells may result in loss and/or altered functions and responses, although some functions may still exist (Matzinger 2007; Matzinger and Kamala 2011). Results can be combined in integrated testing strategies to cover the full process of skin sensitization (Jaworska *et al.* 2011; Jowsey *et al.* 2006; Mehling *et al.* 2012). In the following chapter the most prominent and important steps and corresponding animal-free assays for skin sensitization as well as combined test strategies are described.

2.3.1 *IN SILICO* METHODS

As described in the section “chemical allergens” no specific substance class has been defined as the main cause of skin sensitization, but according to chemical structure and electrophilic properties skin sensitizing potential of test substances can be predicted with the help of algorithms (Patlewicz *et al.* 2003; Roberts *et al.* 2007a). The Roberts assignment (Tabel. 1), described by Roberts and co-workers (Aptula and Roberts 2006; Roberts *et al.* 2007b; Roberts *et al.* 2007c), was successfully applied on 210 substances by Patlewicz and co-workers (Patlewicz *et al.* 2008). They demonstrated that with consideration of mechanistic applicability domains skin sensitizing potential can be predicted. Computer based analysis of molecule structures and experimental data of similar or related structures allows the performance of quantitative structure activity relationships (QSAR). The most relevant *in silico* tools for skin sensitization are the OECD QSAR toolbox (Devillers and Mombelli 2010), Deductive Estimation of Risk from Existing Knowledge (DEREK) (Barratt and Langowski 1999), TIssue MEtabolism Simulator for Skin Sensitization (TIMES-SS) (Patlewicz *et al.* 2007), computer assisted Evaluation of industrial chemical Substances According to Regulation (CAESAR), MCASE and TOP-KAT (Chaudhry *et al.* 2010; Dimitrov *et al.* 2005; Mehling *et al.* 2012; Roberts *et al.* 2007a). None of these test systems has ran

through a formal validation, but have been used for screening purposes (Mehling *et al.* 2012). They can be knowledge based (DEREK), rule based (MCASE and CEASAR), non-rule based (TOP-KAT) or creating relationships between structure toxicity and structure metabolism (TIMES-SS). OECD published a guideline in 2007 in order to verify and to ensure reliability of QSAR test systems (OECD 2007). With the help of *in silico* prediction, structural alerts as well as physico-chemical properties like lipophilicity, expressed as octanol/water partition coefficient, or the bioavailability in the skin can be obtained (Aeby *et al.* 2010; Kasting *et al.* 2008; Roberts and Williams 1982). Additionally modules for theoretical metabolic transformation can be included and allow a prediction of potential pro-haptens and metabolic products, although false positive predictions for non-sensitizing chemicals may be increased. The more comprehensive the data base, the higher the accuracy. Prediction of the sensitizing potential for chemicals with more than one possible reaction mechanism, e.g. aldehydes, proved to be difficult (Franot *et al.* 1994). The reliability and accuracy of such *in silico* models are dependent on the amount and quality of data used to build up the data base.

2.3.2 *IN CHEMICO* METHODS

Reaction between chemical and protein resulting in the formation of stable protein conjugates is an important step in skin sensitization (Landsteiner and Jacobs 1936). Cell free analysis of skin sensitizing potential can be performed using peptides or proteins in so-called *in chemico* methods. With those the skin sensitizing potential according the protein reactivity of chemicals can be predicted (Gerberick *et al.* 2008; Gerberick *et al.* 2007). The most promising *in chemico* assay is the so-called direct peptide reactivity assay (DPRA) described by Gerberick and co-workers in 2004 (Gerberick *et al.* 2004), and is currently undergoing and has nearly competed formal validation at ECVAM (Mehling *et al.* 2012). The test substance is incubated with two artificial peptides, each with an active core containing either one lysine or cysteine residues as nucleophils. The reaction of substance with either peptide is measured as the depletion of the individual peptide by high pressure liquid chromatography with ultra-violet light absorbance detection (HPLC-UV). Reaction of chemicals with peptides result in a shift of the retention time of the respective peptide consequential reducing the peak of the unbound peptide. The areas under the curve of treated peptides and control peptides are evaluated and compared to estimate the amount of conjugated peptide. In 2007 a decision tree was published in which peptide depletion was set in correlation with the potency of skin sensitizers (Gerberick *et al.* 2007). Gerberick and co-workers evaluated the cysteine and lysine dependent peptide depletion as well as the calculated mean of both peptides. They were able to describe thresholds to classify the sensitizing potency of chemicals according the mean peptide depletion. A chemical is rated with minimal reactivity if the calculated mean peptide depletion of lysine and cysteine peptide is smaller than 6.376 % and of low, moderate or high reactivity if the mean peptide depletion is >6.376 but <22.62; >22.62 but <42.47 or >42.47, respectively. Although the DPRA showed good correlation with the skin sensitization data, prediction of chemicals requiring previous metabolic activation cannot be relied on as long as the DPRA does not include a metabolizing system (Gerberick *et al.* 2009; Gerberick *et al.* 2007). To address this Gerberick and co-workers optimized the DPRA by including a peroxide/peroxidase system and showed a reliable prediction of pro-haptens (Gerberick *et al.* 2009). This

improved assays is known as the peroxidase peptide reactivity assay (Gerberick *et al.* 2009; Troutman *et al.* 2011).

Moreover, Natsch and co-workers established a liquid chromatography-mass spectrometry (LC-MS) based detection using a cysteine rich peptide called *Cor1* to detect oxidation of the peptide leading to dimerization via disulfide bonds caused by chemicals and adduct formation between peptide and chemical (Natsch and Gfeller 2008). A prominent example is sodium dodecyl sulfate (SDS). It was shown that SDS caused peptide dimerization and was rated as sensitizer. Accordingly the optimized DPRA with LC-MS analyses increased the predictivity of the peptide depletion by detection of dimerized peptides and is thus able to exclude false positive predicted substances. Further peptide reactivity assays have been published by Aleksic and co-workers, who used several nucleophilic peptides and LC-MS-MS analysis and were able to obtain qualitative and quantitative data as well as results for cysteine dimerisation (Aleksic *et al.* 2008). Schultz and co-workers used GSH in a spectrophotometric assay instead of artificial peptides to predict the protein- or peptide reactive potential of chemicals (Schultz *et al.* 2005). Glutathione (GSH) is a tripeptide consisting of glutamic acid, glycine and cysteine and is omnipresent in cells. GSH has an antioxidative role, especially during oxidative stress and represents a physiological peptide model compared to artificial peptides. This approach was further applied to study the kinetics of haptenization (Böhme *et al.* 2009; Roberts *et al.* 2010). Jeong and co-workers published recently a modified version of the DPRA in which HPLC-UV analysis of unbound peptides was replaced with a colorimetric or fluorescent analysis. After incubation with chemicals the peptides are treated with either amine or thiol reactive agent, which only binds to non-conjugated peptides and indicates whether the chemical was able to bind to the peptide. Although this method does not reach the predictivities of the DPRA yet, it offers a high-throughput method and is independent of HPLC devices (Jeong *et al.* 2012).

2.3.3 *IN VITRO* METHODS

After the formation of complete antigens (the complexes between haptens and peptides or proteins) cellular responses in the skin are required for an immune response (Kimber *et al.* 2002a; Kimber *et al.* 2002b; Kimber and Dearman 2002)

2.3.3.1 KERATINOCYTE RESPONSE

As the main cell type in the epidermis, KCs elaborate inflammatory responses and thus facilitate the activation of DC via the release of cytokines like IL-1α or IL-18 (Antonopoulos *et al.* 2008; Cumberbatch *et al.* 2001; Van Och *et al.* 2005). KC are metabolically capable cells and are the main activator of pro-haptens into protein reactive metabolites (Bergstrom *et al.* 2007; Jaeckh *et al.* 2011; Oesch *et al.* 2007; Reilly *et al.* 2000; Rolsted *et al.* 2007; Roychowdhury and Svensson 2005; Svensson 2008), in addition to this it was shown that the presence of pro-haptens induce levels of CYP1A1 mRNA in KC and thus increased the level of this enzyme, a monooxygenase of the cytochrome P450 familiy and transforms its substrate into epoxides (Al Masaoudi *et al.* 2001; Oesch *et al.* 2007). *In vitro* assays based on human or murine KC cell lines, like HaCaT, NCTC2544 or HEL-30, have been used to detect cytokine release (Corsini *et al.* 1998; Corsini *et al.* 1999; Corsini *et al.* 2009;

Galbiati *et al.* 2011; Van Och *et al.* 2005) or intracellular pathways (Natsch and Emter 2008) in response to sensitizers. Treatment of KC with skin sensitizer showed a dose dependent increase of released IL-1α and/or IL-18 whereas irritants, such as salicylic acid, lactic acid and SDS, did not induce cytokine release (Corsini *et al.* 2009; Van Och *et al.* 2005). The signaling pathway known as Kelch-like ECH associated protein-1 -Nuclear factor (erythroid derived 2)like 2 (Keap1-Nrf2) pathway was shown to be one of the signaling pathways involved in skin sensitization and was highly investigated in the last years (McKim *et al.* 2010; Natsch and Emter 2008). The Keap1-Nrf2-pathway is described as a cellular sensor for oxidative stress as well as electrophilic substances (Dinkova-Kostova *et al.* 2005; Wakabayashi *et al.* 2004; Wang and Jaiswal 2006). In unstressed cells the transcription factor Nrf2 is bound to the protein Keap1 and thus not able to translocate into the nucleus. In addition, if bound to Keap1, Nrf2 will be degraded after ubiquitinylation. Oxidative stress and consequential reactive oxygen species (ROS) and/or electrophilic chemicals activate proteins such as protein kinase C which phosphorylates Nrf2 and cysteine reactive electrophiles bind to free cysteine residues of Keap1. In consequence of the phosphorylation of Nrf2 and changes to Keap1 conformational changes of proteins occurs which triggers the dislocation of Keap1 and the translocation of Nrf2 into the nucleus. Nrf2 binds to antioxidant response elements (ARE) and regulates gene expression of so-called Phase 2 enzymes like epoxide hydrolase, quinine reductase 1 and glutathione s transferase under the control of ARE. The up-regulation of those enzymes counteract ROS and triggers the reduction of electrophiles (Dinkova-Kostova *et al.* 2005; Wakabayashi *et al.* 2004; Wang and Jaiswal 2006). A correlation between the activation of Keap1-Nrf2-ARE and the treatment with skin sensitizers was shown and reporter gene cell lines to monitor Keap1-Nrf2 pathway activation were created (Ellis *et al.* 2009; Emter *et al.* 2010; Natsch and Emter 2008). Keratinocyte reporter gene cell lines, KeratinoSens and LuSens, both carrying plasmids with an ARE element of the human aldoketo reductase gene AKR1C2 (KeratinoSens) or the rat NADPH:quinone oxidoreductase 1 gene (LuSens) followed by a reporter gene encoding luciferase (Bauch *et al.* 2012; Emter *et al.* 2010). Although a good correlation of sensitizers with the activation of Keap1-Nrf2 pathway was found, some sensitizers fail to activate Nrf2 regulated gene expression and activate different pathways instead. A prominent example is nickel, which was described by Ade and co-workers to activate NFκB signaling but not Nrf2. Nickel forms to coordinative bonds only and is thus not able to form the required covalent bonds to cysteine (Ade *et al.* 2007).

2.3.3.2 DENDRITIC CELL ACTIVATION

iDC patrol and screen the skin for foreign molecules (Banchereau and Steinman 1998; Ryan *et al.* 2007; Steinman 1991; Steinman 2001). As APC they are able to recognize, process and present those molecules on their cell surface mainly to T cells. APC of the epidermis are called LC and DC in other tissues (Toebak *et al.* 2009). DC respond to changes in their environment and are able to detect foreign, exogenous molecules like as bacterial structures, such as lipopolysaccharide or glycoproteins, or proteins like ovalbumin and chemicals (Aiba *et al.* 1997; Cella *et al.* 1997; Hulette *et al.* 2002 Ryan *et al.* 2007; Steinman 1991). The process of antigen recognition and DC activation is complex and several parameters are involved and some aspects are still unknown. It has not been identified, yet, how DCs recognize hapten and/or hapten-protein conjugates. A common target, such as a receptor, might be unlikely due to the variety in chemicals structures and the

unspecific electrophilic reactions of the haptens with a variety of proteins (OECD 2012). Different theories described how dendritic cell may take up haptens. DC possess endocytically activity (Steinman 2001) and are able to take up hapten-protein conjugate and to process them (Banchereau and Steinman 1998; Steinman 2001). The uptake may happen non-specifically via endocytosis or via internalization of changed cell surface proteins and in addition it was shown that haptens or hapten-protein conjugates trigger different signaling pathways (Ade *et al.* 2007; Neves *et al.* 2011; OECD 2012). Moreover, active transport or passive diffusion through the membrane may take place (Smith and Hotchkiss 2001; Smith-Pease 2003) and it was shown that several toll like receptors (TLR), P2X7-dependent inflammasome, ROS and the degradation of extracellular matrix seem to play a role during the process of recognition and uptake of chemicals allergens, but interactions vary for different chemicals (Martin *et al.* 2008; Schmidt *et al.* 2010; Weber *et al.* 2010).

Nevertheless, DC recognize allergens in their environment, leading to their activation and maturation (Ryan *et al.* 2007). Mature DC migrate out of the epidermis towards the local lymph node (Larsen *et al.* 1990). They encounter T cells and present the antigen on their cell surface (Kimber *et al.* 2002a; Macatonia *et al.* 1987). The expression of several maturation markers and the release of cytokines are generally correlated with treatment of sensitizing chemicals (Aiba *et al.* 1997; Ozawa *et al.* 1996; Rambukkana *et al.* 1995). It was shown that DC release cytokines like IL-1β or TNFα, and express cell surface proteins like CD86, CD80, CD54 and CD40 (Enk and Katz 1992; Hulette *et al*. 2002 Ozawa *et al.* 1996; Pichowski *et al.* 2000; Tuschl *et al.* 2000; Tuschl and Kovac 2001; Verrier *et al.* 1999). CD40, CD80 and CD86, so called co-stimulatory molecules, bind to their counterparts CD40L or CD28 (ligand for CD80 and CD86) on T cells. It is assumed that CD86-CD28 interaction amplifies the activation of T cells by mature DC (Fagnoni *et al.* 1995) and the CD40-CD40L interaction is known to trigger IL-12 release and T cell stimulation (Cella *et al.* 1996). CD54, an intercellular adhesion molecule (ICAM-1), is expressed by endothelial cells and immune cells such as DC, and has an important role in cell migration. It was shown that CD54 is up-regulated after exposure of DC to sensitizer (Ozawa *et al.* 1996).

To simulate the cellular response of skin sensitization *in vitro* different sources of cells are available. Primary DC can be freshly isolated from skin, either human or pig skin. In addition monocytes isolated from peripheral or cord blood can be used to differentiate into DC. Different kind of immortalized cell lines are available to reflect DC, e.g. THP-1, U-937, MUTZ-3 or KG-1 (Mehling *et al.* 2012). Primary cells provide a system close to the *in vivo* situation but unfortunately the isolation of DC from skin has been proven to be difficult. Not only is the frequency of DC in the skin low with a percentage of 1-3 % of total epidermal cells (Bauer *et al.* 2000) also is the rate of spontaneous maturation triggered by the isolation procedure relatively high (Hanau *et al.* 1988; Peiser *et al.* 2003; Teunissen *et al.* 1988). Useful surrogates for epidermal DC have been found using human CD14 positive peripheral mononuclear blood cells (Lenz *et al.* 1993; Reuter *et al.* 2011; Sallusto and Lanzavecchia 1994) and human CD34 positive hematopoietic progenitor cells isolated from cord blood or bone marrow (Caux *et al.* 1997; Hooyberghs *et al.* 2008; Inaba *et al.* 1992). Those cells can be differentiated into DC using cytokines like IL-4 or granulocyte macrophage colony-stimulating factor. Obtaining a DC like phenotype after differentiation those cells can be used instead of epidermal DC for *in vitro* test systems, although they offer

high donor-to-donor variations and the procedures are expensive and time consuming (dos Santos *et al.* 2009). Additionally, the handling of potentially infectious human material might be a limiting factor of use (Mehling *et al.* 2012). Therefore the availability of stable DC-like cell lines such as monocytic cell line THP-1 (Ade *et al.* 2009; Ashikaga *et al.* 2002; Sakaguchi *et al.* 2006; Yoshida *et al.* 2003), myeloid cells lines U-937 (Ade *et al.* 2009; Python *et al.* 2007) and MUTZ-3 (Azam *et al.* 2006; Johansson *et al.* 2011; Python *et al.* 2007), the bone marrow derived cells KG-1 offered a convenient option for *in vitro* test systems. Those cell lines have been intensively studied in the last years and showed DC like characteristics such as a phenotype, properties like endocytosis and maturation after treatment with lipopolysaccharide. In addition cell lines ensure a stable maintenance of the cells in culture and as well more stable phenotype compared to primary cells. Upon stimulation with microbial endotoxins, such as lipopolysaccharides or glycoproteins, or sensitizing chemicals, the above mentioned cell lines showed changes in cell surface markers being characteristic for the process of skin sensitization, upregulation of CD86, CD54, CD40 and MHC molecules as well as the release of cytokines like IL-1β (Ashikaga *et al.* 2002; Azam *et al.* 2006; Enk and Katz 1992; Lepoittevin *et al.* 1998; Python *et al.* 2007; Yoshida *et al.* 2003). Two methods for cell activation of DC like cells turned out to be promising. The human cell line activation test (h-CLAT) and the myeloid U-937 skin sensitization test (MUSST) have been submitted for pre-validation at ECVAM. Pre-validation of the h-CLAT is nearly finished (Mehling *et al.* 2012), whereas the MUSST pre-validation was stopped or postponed. The h-CLAT is performed using THP-1 monocytes and was developed by researchers at the companies KAO and Shiseido (Ashikaga *et al.* 2006; Nukada *et al.* 2011a; Nukada *et al.* 2012; Sakaguchi *et al.* 2006; Sakaguchi *et al.* 2007). Within this test, THP-1 cells are exposed to chemicals in various concentrations for 24 h and are analyzed regarding their expression of CD86 and CD54 cell surface markers using antibody staining and flow cytometry. If the expression of at least one of these markers is increased (> 150 % for CD86 and > 200 % for CD54) compared to control cells, the chemical is rated as a skin sensitizer. The h-CLAT was intensively studied to proof reliability and performance of the method and to investigate its predictivity (Ashikaga *et al.* 2010; Nukada *et al.* 2011a; Nukada *et al.* 2011b; Sakaguchi *et al.* 2006; Sakaguchi *et al.* 2009; Sakaguchi *et al.* 2010). The MUSST assay is a similar method compared to the h-CLAT. U-937 cells are used as DC like cells and CD86 is analyzed as maturation marker. This method was initially developed by Python and co-workers at Procter and Gamble (Python *et al.* 2007) and was further established as the so called MUSST by scientists at L´Oréal (Ade *et al.* 2006). Cells are incubated with chemicals for 48 h and then analyzed regarding their CD86 cell surface expression using antibody staining and flow cytometry. A substance is rated as skin sensitizer if the expression of CD86 was increased compared to control cells. The threshold was set for a fold induction of 1.2, comparable with a 20 % increase. Several studies have been conducted to show predictivity, reproducibility and robustness of the MUSST (Aeby *et al.* 2010; Maxwell *et al.* 2011; Thyssen *et al.* 2007). The changes in cell surface protein expressions are important indicators for activated DC. Nevertheless, gene expression using microarray-based technology showed promising results (Hooyberghs *et al.* 2008; Roggen 2011; Schoeters *et al.* 2007). In the past two microarray based assays have been established, the VITOSENS and the "genomic allergen rapid detection test", GARD assay (Hooyberghs *et al.* 2008; Johansson *et al.* 2011; Lambrechts *et al.* 2011; Lambrechts *et al.* 2010b; Lambrechts *et al.* 2010a). VITOSENS is performed using CD34 positive monocytes derived from

human cord blood which is collected straight after birth with permission of the women. In order to substitute cord blood derived cell to be independent from donors and to reduce donor-to-donor variability, the assay was adopted to use the cell line THP-1 instead. Although THP-1 cells were able to discriminate sensitizer and non-sensitizer it was shown that THP-1 cells respond to sensitizer with different intracellular cascades than CD34 positive monocytes. Despite the convenience of the cell line the primary cell model remained as cell type of choice (Lambrechts *et al.* 2009). The second assay is the GARD assay using MUTZ-3 cells as DC surrogates (Johansson *et al.* 2011). Cells were treated for 24 h with the chemicals and the transcriptome or RNA content were analyzed and a biomarker signature of 200 genes were identified showing potent ability to discriminate sensitizers and non-sensitizer (Johansson *et al.* 2011).

Another noteworthy method is a transwell migration assays using MUTZ-3 cells differentiated into Langerhans' cell surrogates. DC are able to migrate along chemokine gradients either into the skin or outwards. With regards to exposure to chemical sensitizers activation of DC leads to a shift in their chemokine receptor expression on the cell surface and thus a changed response to chemokines (Ouwehand *et al.* 2010). The idea is to discriminate sensitizers and non-sensitizers according the migration DC (dos Santos *et al.* 2009). Immature DC, or after treatment with non-sensitizer, migrate along a skin homing chemokine, e.g. CCL5, whereas mature DC, or after exposure to sensitizer, migrate towards CXCL12, a lymph node homing chemokine. This was implemented in an *in vitro* assay using MUTZ-LC and recombinant chemokines. Ouwehand and co-workers showed a correlation between DC treated with sensitizer and their characteristic to migrate towards CXCL12 and between DC treated with non sensitizer and the migration towards CCL5. In addition, they proved the maturation state of DC by analyzing the CD86 expression and the secretion of CXLC8 (Ouwehand *et al.* 2010).

2.3.3.3 CO CULTURE AND SKIN MODELS

The function and activity of cells in single cell cultures may be different from mixed cultures or in their physiological environment (Matzinger 2007; Matzinger and Kamala 2011). It was shown that single cell cultures provide satisfactory results, but nevertheless failed to predict the sensitizing potential of certain substances, especially pro-haptens (Chipinda *et al.* 2011). The interactions of KC and DC facilitate the response and activation of DC, thus co-cultures of both cell types may allow cell-cell interactions to resemble the *in vivo* situation. Such co-cultures could take advantage of the metabolic capacity provided by KC to activate pro-haptens in order to identify potential pro-haptens (Hennen *et al.* 2011). Schneider and co-workers have combined primary human KC with human monocyte derived DC in the "loose fit co-culture based sensitization test (Schreiner *et al.* 2007). DC are evaluated according their maturation state and the expression of CD86 to identify the sensitizing potential of chemicals. A dose-response analysis indicates the potency of the sensitizer. In addition a co-culture completely devoid of primary cells was published by Hennen and co-workers (Hennen *et al.* 2011). In their studies it was shown that the combination of the KC cell line HaCaT and the DC cell line THP-1 seemed to be promising for the prediction of haptens and pro-haptens. The activity of CYP1 enzymes, necessary for metabolic conversion of pro-haptens, was increased in co-culture treated HaCaT cells in the presence of pro-haptens, e.g. eugenol (Hennen *et al.* 2011). In addition co-cultures with reconstructed epidermis

and LC (Facy *et al.* 2004; Facy *et al.* 2005) or full-thickness skin model with MUTZ-3 cells (Laubach *et al.* 2011) have been described. Co-culture systems, regardless if DC are cultured with KC cell lines or skin models, provide cell-cell interactions similar to the skin. Culture conditions have to be adapted carefully to not influence the growth of one or all cells types and to avoid reduced responsiveness. In addition it has to be monitored and proved that DC are not activated during co-culture to avoid false positive results.

As mentioned above reconstructed human epidermis (RHE), also known as skin models or skin equivalents, are available and represent artificial, 3D-structured cultures of KC. Various models are commercially available and are used to assess chemicals with respect to skin irritating potential (Skinethic™, Epiderm™ and Episkin™) (Mehling *et al.* 2012). Metabolic competence was shown for these skin models (Gibbs *et al.* 2007; Jaeckh *et al.* 2011) and are thus applicable to predict pro-haptens in the above mentioned co-cultures. The available skin models are not immunologically competent yet due to lack of APC or immune cells, thus no differentiation between sensitizers and irritants can be performed (dos Santos *et al.* 2011) However, it was shown that cell viability and release of cytokine IL-1α is in correlation with the irritating potential of the test substance. With the implementation of skin models in so-called two-tiered testing strategies the prediction of the skin sensitizing potential using e.g. a DC activation assay, and a potency analysis using the skin models is feasible (dos Santos *et al.* 2011; McKim *et al.* 2010; Teunis *et al.* 2012). Another model was described by Uchino and co-workers. Here, a 3D co-culture of DC, KC and fibroblasts represents a reconstructed skin model with the help of vitrigel collagen as the 3D matrix. Analysis of cell surface markers and cytokines after treatment with several chemicals sensitizers could be identified. Thus, not only the advantages of cell-cell-interactions and possible cross talk of different cell types is combined in this model but also the immune-competence obtained by DC (Uchino *et al.* 2009).

2.3.3.4 T CELL ACTIVATION

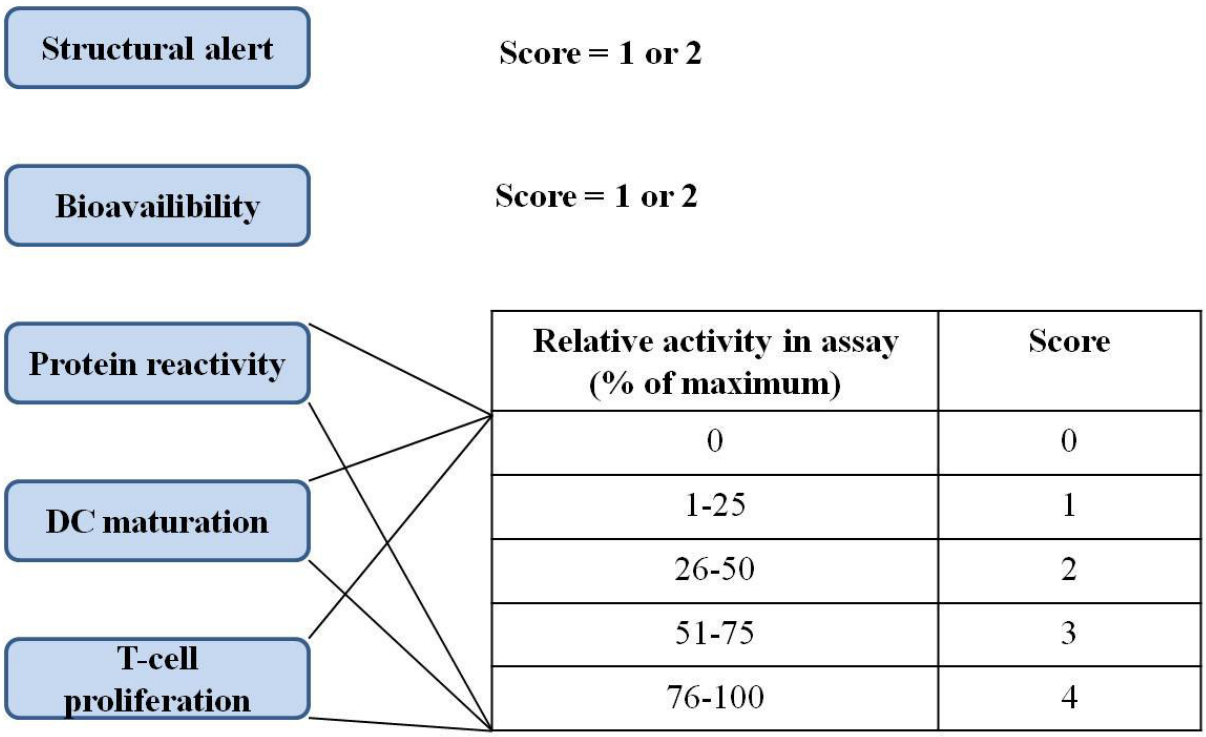
Activation of naïve T cells to antigen specific memory cells is the last step in the skin sensitization process (Kimber *et al.* 2002a; Thierse *et al.* 2004). This step requires the interaction of T cells with mature DC, presenting the antigen via MHC class II molecules on their cell surface (Kimber and Cumberbatch 1992). Several protocols to mimic T cell activation have been published over the last years (Dai and Streilein 1998; Dietz *et al.* 2010; Guironnet *et al.* 2000; Krasteva *et al.* 1996; Moulon *et al.* 1993; Rougier *et al.* 2000; Rustemeyer *et al.* 1999) measuring the proliferation of naïve T cells upon allergen treatment. However, these protocols are not advanced enough to discuss their implementation in testing strategies (Martin 2012; Mehling *et al.* 2012). The development of T cell based *in vitro* assays is confronted with challenges. Every individual person possesses a pool of naïve T cells, each carrying a specific TCR for one antigen. It was reported that some allergens may have more than one TCR counterpart, however in each T cell pool only about 1 % of cells have the required specificity for the tested allergen. T cell response *in vitro* requires always primary source of T cells as stable cell lines are usually derived from one clone entailing the lack of TCR variety (Kimber *et al.* 2011; Moon *et al.* 2007).

2.3.4 TEST STRATEGIES

Integrated testing strategies (ITS) or assay combinations are required if complex *in vivo* processes like skin sensitization have to be simulated by *in vitro* assays (De Wever *et al.* 2012; Jowsey *et al.* 2006). Combination of assays allows a reflection of the whole process, albeit performed in independent, complementary assays (Basketter and Kimber 2009; Jowsey *et al.* 2006). In the past several combinations of assays have been described in which different steps of skin sensitization have been covered or included. Jowsey and co-workers proposed a point system based model which served as basis for the development and combination of following test strategies (Jowsey *et al.* 2006). This model by Jowsey and co-workers describes an example for a so-called integrated testing strategy (ITS) to predict not only the skin sensitizing potential (skin sensitizer Yes/No) of chemicals but also their potency (classification between weak, moderate, strong and extreme). Five steps were included in this strategy: (1) structural alerts, (2) bioavailability, (3) protein reactivity, (4) DC maturation and (5) T-cell proliferation (Fig. 3). Each individual test is rated with a score between 0 and 4. The parameters structural alert and bioavailability are scored with 2 for a clear structural alert or bioavailability. In any other cases or uncertainties a score of 1 is assigned as a zero score would imply the lack of potential to induce sensitization. The multiplication of scores results in an index of sensitizing potency (Jowsey *et al.* 2006). According to this proposal several combinations and test strategies have been described. Natsch and co-workers described a test battery combining peptide reactivity, induction of ARE-dependent luciferase activity in a cell-based assay, *in silico* prediction using TIMES-SS, and calculated octanol/water partition coefficient. The predictivity of this test battery was 87 % sensitivity, 81 % specificity and 85 % accuracy for number of 116 test chemicals (Natsch *et al.* 2009). Another approach was published by the US contract research organization CeeTox, who are specialized with *in vitro* screenings of drugs and chemicals. A semi-quantitative method to determine sensitizing potency was described and combines glutathione peptide depletion with data from gene expression analysis of Keap1/Nrf2/ARE/EpRE, ARNT/AhR/XRE or Nrf1/MTF/MRE pathways regulated genes was applied to 67 substances and indicated a sensitivity of 81 % and a specificity of 92 % (McKim *et al.* 2010). A probabilistic approach was proposed as Bayesian network integrated testing strategy (BN ITS) combining *in silico* (several parameters), *in chemico* (DPRA) and *in vitro* data (ARE luciferase activity, MUSST and IL-8 secretion) to estimate skin sensitization hazard (Jaworska *et al.* 2011). It was concluded that simple combinations of test methods connected to a decision tree are unlikely to provide an effective display of the data assessment and further that the choice and order of tests are dependent on available information and the chemical itself.

Noteworthy is the concept of Threshold of Toxicological Concern (TTC). This concept can be used if no experimental toxicological data are existing but knowledge about chemical structure and exposure limits are given. Thresholds can be created to describe negligible risk to human health using statistical analysis of toxicological data from different and/or structurally related chemicals and extrapolation of no-effect dose levels from underlying animal data (Kroes *et al.* 2005). According to TTC two additional tiered approaches have been published, the dermal sensitization threshold (DST) based on existing LLNA data (Safford 2008; Safford *et al.* 2011) and the threshold of sensitization concern based human patch test data (TSC; (Keller *et al.* 2009)). If TSC

shows that estimated human exposure exceeds the TSC a QSAR analysis is suggested. In case of negative QSAR results DPRA is suggested and a MUSST or h-CLAT assay should be performed in case of uncertainties (Keller *et al.* 2009). In the work presented here a similar, to the above mentioned, test strategy is described (see Chapter 6). Combination of protein reactivity (DPRA), KC activation (KeratinoSens assay) and DC like cell activation (MUSST or h-CLAT) is described and showed either high probable prediction of non-sensitizers with the combination of DPRA and a reporter gene analysis of ARE related genes, and of sensitizers using the MUSST and a high overall accuracy with 94 % by combing all three assays (Bauch *et al.* 2012).



Relative activity in assay (% of maximum)	Score
0	0
1-25	1
26-50	2
51-75	3
76-100	4

Figure 3: Integrated testing strategy for skin sensitization (Jowsey et al. 2006)
A binary score was used for data related to structural alerts (derived, for instance, from DEREK) and skin penetration potential. A score of 2 is given if structural alerts were identified unequivocally, or if a clear potential of skin bioavailability was identified. In all other cases data would be scored 1. For the three *in vitro* experimental data sets, values between 0 and 4 are assigned based on the relative activity compared to a model compound of known high sensitizing potential

3 AIM OF THE THESIS

Animal-free methods to predict the hazard of chemicals such as skin sensitizing potential are becoming a more and more important issue due to public interest in animal welfare and demands by legal bodies, such as the European Union. The process of skin sensitization comprises several steps which are unlikely to be sufficiently reflected in a standalone alternative method implying that several assays have to be developed to rebuild the process of sensitization *in vitro.* Before such assays can be used to test chemicals regarding their safety, the validity and reliability of these tests have to be confirmed by formal validation initiated by governments and within each laboratory performing these assays.

The overall purpose of this work was to establish cell based *in vitro* assays and to validate them in order to use them as valid screening test systems. The first part on the validation of several animal-free, cell based assay was to be performed at BASF SE toxicology laboratories in Germany and included to show the reliability and accuracy of these assays and as well to indicate weak points, such as changes of responsiveness of cells over time or limitations of positive or negative control substances. A total of 54 substances were selected with known sensitizing potential and available human patch test data and/or LLNA from the literature. Furthermore, after successful validation test batteries or test strategies should be described to combine several assays to reflect the process of skin sensitization and to increase the validity for the detection of probable skin sensitizers.

1) Four cell based assays were selected for validation: two DC-like cell activation tests (MUSST and h-CLAT) with the flow cytometric analysis of proteins on the cell surface, and two tests based on reporter gene cell lines indicating activated Nrf2-ARE-signaling pathway with luminescence as readout for activated gene expression. In addition cell viability tests were to be performed in parallel using propidium iodide staining or the proliferation assay MTT. Additional data from the peptide binding assay DPRA and computer based analysis of molecule structures using the OECD toolbox were already available and evaluated for purposes of creating a test strategy.
2) By means of Cooper statistics for each assay the predictivities should be expressed with values for sensitivity, specificity and accuracy to be able to directly compare the assays and to identify most meaningful assay combinations in order to reflect the process of skin sensitization in its complexity and to increase the predictivity compared to single assays.

Since the first part indicated a high predictive value of those assays which address early protein interactions of haptens, the second part further investigated protein reactivity and the interference of antibody binding to proteins caused by the haptenization. To gain better insights into the process of haptenization and its significance in skin sensitization and compound validation mechanistic studies should be carried out. Therefore, the second part of this thesis was performed in the Laboratories of the Toxicology group at the University of Manchester in a group specialized on skin sensitization. This work comprised the experimental study of protein reactivity of chemicals with proteins on the cell surface and the characterization of interference of these

haptenizations with antibody binding to those proteins on the cell surface. Focus was set on the investigation of following aspects:

1) With the model sensitizer 1-chloro-2,4-dinitrobenzene (DNCB) and the model cell surface proteins, CD14 and CD54, it should be determined whether treatment of cells with sensitizers results in reduced binding of antibodies against those proteins or if chemical treatment leads to reduced levels of detectable proteins due to protein internalization. Antibody staining and flow cytometric analyses were performed on viable and metabolically inhibited cells to show the influence of cell viability on results.
2) To investigate whether proteins with DNCB are internalized or if DNCB is able to permeate through the cell membrane, intracellular bound DNCB should be detected after permeabilization of cells with saponin and intracellular antibody staining by flow cytometric analysis.
3) Further it was to be assessed whether additional sensitizers or irritants have the same effects on detectable cell surface proteins as DNCB. Therefore *para*-phenylene diamine, *para*-benzoquinone, oxazolone and formaldehyde were to be used as sensitizers and SDS and salicylic acid as irritants.

4 VALIDATION STUDY OF THE KERATINOSENS ASSAY

4.1 THE INTRA- AND INTER-LABORATORY REPRODUCIBILITY AND PREDICTIVITY OF THE KERATINOSENS ASSAY TO PREDICT SKIN SENSITIZERS *IN VITRO*: RESULTS OF A RING-TRIAL STUDY IN FIVE LABORATORIES

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The KeratinoSens assay is a reporter gene based analysis to predict skin sensitizing potential of chemicals. The cell line used in this assay shares the same name than the assay: KeratinoSens. The cell line was developed by Natsch and co-workers (Ellis *et al.* 2009; Emter *et al.* 2010; Natsch and Emter 2008). The human keratinocyte cell line HaCaT was stably transfected with a plasmid carrying an ARE element of the *AKR1C2* gene and a reporter gene for the firefly luciferase. The enzymatic activity of luciferase was used for readout. Genes under the control of ARE have been shown to be up-regulated if cells were exposed to sensitizer to regulate glutathione homeostasis, inhibition of inflammation and the depletion of ROS (Dinkova-Kostova *et al.* 2005). The mediator of ARE activation is the transcription factor Nrf2. In non-stressed cells Nrf2 is inactive as it is bound to the protector protein Keap1 and degraded after being ubiquitinylated. Oxidative stress and consequential ROS or electrophilic substances, such as skin sensitizers, activate the phosphorylations of Nrf2 by kinases such a protein kinase C. the Keap1 protein is rich in free cysteine residues and is thus a potential reaction partner of cysteine reactive electrophiles. Conformational changes caused by the phosphorylations of Nrf2 and the reaction with cysteine residues of Keap1 leads to the dissociation of Nrf2 and Keap1 and thus promotes the translocation of Nrf2 into the nucleus. It was shown that skin sensitizers are able to specifically activate the Nrf2-ARE-gene expression (Natsch and Emter 2008; Ryan *et al.* 2004; Vandebriel *et al.* 2010) although some sensitizer, like nickel, fail to activate it (Ade *et al.* 2007).

The ring trial study presented in this manuscript was initiated and led by Givaudan, a fragrance producing company in Switzerland. The purpose was to show that the *in vitro* assay KeratinoSens is transferable and reproducible. In addition one intention was to identify critical steps in the standard operating procedure and to set quality criteria in order to improve the protocol for a successful submission for formal pre-validation at ECVAM.

Cells were maintained under the presence of the selection marker Geneticin to ensure the propagation of reporter gene positive cells. For chemical treatment, the adherent cells were trypsinized and plated in 96 well

plates for luminescence analysis or normal cell culture plates for cell viability assay and were allowed to adhere for 24 h. Chemicals were dissolved in appropriate solvent and medium and cells were treated for 48 h. Induction of the reporter gene was read by measuring the activity of the luciferase via the emitted luminescent signal upon addition of coelenterazine. Cytotoxicity caused by tested substances was measured using a proliferation and cell viability assay with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, the MTT cell viability assay.

The study comprised two phases. During the first phase the correct performance of the protocol, the stability of the cell line and sensitivity of the used luminometer reader of each laboratory were to be proved. The protocol was easily adopted in each laboratory although no training was performed. Two hurdles were reported, although they were more of technical nature than related to the assay and cell line. Different luminometer readers and luciferin substrates led to variations in background levels between the laboratories. Diverse luciferase substrates, namely flash-substrates and glow-substrates, exhibited differences in the absolute dose of emitted light. Glow-substrates are adopted for high-throughput screenings and ensure a steady, long-lived signal, entailing a reduced absolute amount of emitted light. Flash substrates to the contrary provide a strong emitted light signal, which quickly decreases over time. For the purpose of the ring trial study the flash substrate was selected in order to increase the sensitivity of the assay.

Within the first phase of the ring trial study four sensitizer and three non-sensitizers were tested and proved the stable performance of the assay in each laboratory, and that variations between laboratories do not affect the prediction. Three of four sensitizers were identified as sensitizers and the dose response curves were comparable. All non-sensitizers were reported with negative response, whereas one sensitizing substance showed borderline behavior.

The second phase of the ring trial study comprised a set of 21 blind-coded substances; among which 15 substances were known skin sensitizer and the other six non-sensitizers. Thus, with the seven substances tested in phase 1, each participant tested a total number of 28 substances. The calculations of Cooper statistics indicated sensitivities greater than 84 % in all laboratories with the highest sensitivity amounting to 94 %. Specificities were 100 % for all laboratories except one (78 %) and the accuracies were greater than 86 %. Despite the high predictivity false negative substances were reported.

Overall, the ring-trial study indicated that the KeratinoSens assay is reliable in its performance and yields high predictivity, at least for the limited number of tested substances. The inter-laboratory transfer of the assay and the protocol indicated few, technical issues, which were addressed by including a training protocol to ensure parameters such as high sensitivity, low variability in controls and exclusion of gradients across the plate especially caused by the plate reader used for luminescence measurements. Quality criteria were adjusted and the new, updated protocol was submitted for official pre-validation at ECVAM.

This validation study was part of my PhD project. The establishment of the assay within our laboratories as well as the performance of all the experiments of one participating laboratory (Lab 3) and the data evaluation have been solely performed by myself.

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The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers *in vitro*: Results of a ring-study in five laboratories

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ABSTRACT

Due to regulatory constraints and ethical considerations, research on alternatives to animal testing to predict the skin sensitization potential of novel chemicals has gained a high priority. Accordingly, different *in vitro*, *in silico* and *in chemico* approaches have been described in the scientific literature to achieve this goal. To replace regulatory approved animal tests, these alternatives need to be transferable to other labs, their within and between laboratory reproducibility must be assured, and their predictivity should be high. The KeratinoSens assay is a cell-based reporter gene assay to screen substances with a full dose–response assessment. It is based on a stable transgenic keratinocyte cell line. The induction of a luciferase gene under the control of the antioxidant response element (ARE) derived from the human *AKR1C2* gene is determined. Here we report on the results of a ring-study with five laboratories performing the KeratinoSens assay on a set of 28 test substances. The assay was found to be easily transferable to all laboratories. Overall both the qualitative (sensitizer/non-sensitizer categorization) and the quantitative (concentration for significant gene induction) results were reproducible between laboratories. A detailed analysis of the transferability, the within- and between laboratory reproducibility and the predictivity is presented.

1. Introduction

Cosmetic legislation in Europe has imposed a ban on animal testing for the detection of the skin sensitization hazard of cosmetic ingredients by 2013. Therefore, predictive *in vitro* tests are urgently needed to maintain the ability of the industry to invent new products and to guarantee their safety after 2013. At the same time, the REACH regulation (registration, evaluation, authorization and restriction of chemicals) requires the testing of thousands of not previously tested substances for their skin sensitization potential, thus increasing animal testing. Currently, the skin sensitization potential is estimated with the local lymph node assay in mice (LLNA), in which the cellular proliferation in the draining lymph nodes is measured after repeated topical application of the test substance onto the ears. Results are expressed as EC3 values which indicate the concentration which induces a threefold increase in lymph node cell proliferation as measured by ^{3}H-thymidine uptake (Basketter et al., 2002).

Skin sensitization is an immune reaction to small exogenous molecules. In general, skin sensitizing molecules are reactive chemicals (or chemicals metabolically transformed into reactive intermediates) which have the potential to react with skin proteins

Abbreviations: LLNA, local lymph node assay; Nrf2, nuclear factor-erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; SOP, standard operating procedure; WLR, within laboratory reproducibility; BLR, between laboratory reproducibility; PC, predictive capacity; ECVAM, European centre for the validation of alternative methods to animal testing; ICCVAM, interagency coordinating committee on the validation of alternative methods; RT-PCR, reverse transcriptase polymerase chain reaction; DMSO, dimethylsulfoxide; DNCB, 2,4-dinitrochlorobenzene; SLS, sodium lauryl sulphate; MCI, (5-Chloro)-methyl-isothiazolinone; IC50, inhibitory concentration for 50% reduction in viability as determined with the MTT assay; EC 1.5, extrapolated concentration for 1.5-fold luciferase induction above threshold; REACH, registration, evaluation, authorization and restriction of chemicals; h-CLAT, human cell line activation test; MUSST, myeloid U937 skin sensitization test.

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and render them immunogenic (Karlberg et al., 2008). In the sensitization phase, the modified proteins are processed by dendritic cells and presented in the local lymph nodes, where they provoke the expansion of allergen-specific T-cell clones. Upon repeated contact with the skin sensitizer in the elicitation phase, a specific T-cell mediated immune response then leads to the disease status known as contact allergy (Kimber et al., 2002).

Although this complex cascade of events is difficult to model, three non-animal approaches are in advanced development. It is thought that a combination of these three approaches may finally replace the current animal tests (Jowsey et al., 2006; Basketter and Kimber, 2009; Natsch et al., 2009): (i) *In silico* models predict the sensitization potential of chemicals based on a number of rules or structural alerts empirically derived from databases on known skin sensitizers (Roberts et al., 2007). (ii) *In chemico* models determine the sensitization potential based on an assessment of the chemical reactivity of the test chemicals with nucleophiles, normally model peptides (Gerberick et al., 2008). (iii) Lastly, the cell-based *in vitro* models evaluate certain aspects of the cellular response to skin sensitizers. Most of these latter assays address the unspecific innate response of the skin to sensitizing agents rather than the specific T-cell response.

The *in vitro* assays usually use dendritic cells or keratinocytes, the two cell types in first contact with the topically applied sensitizers. The specific markers investigated in these cells may be selected either at the protein level or at the transcription level. At the protein level, the surface markers CD86 and CD54 and the chemokine IL-8 and IL-18 have been investigated in most detail as predictive markers (Aeby et al., 2004; Toebak et al., 2006; Corsini et al., 2009). Based on these studies, the h-CLAT (Ashikaga et al., 2006) and MUSST *in vitro* assays, which measure surface marker expression in THP-1 and U-937 cells, respectively, have entered pre-validation.

At the transcription level, a number of research groups have reported novel bio-markers for skin sensitizers. Most studies at the transcription level have been based on gene chip analysis of primary cells exposed to sensitizers or irritants (Ryan et al., 2004; Hooyberghs et al., 2008; Python et al., 2009). Several research groups then followed up these initial studies with RT-PCR analysis of the identified markers (Gildea et al., 2006; Python et al., 2009). However, attempts to transfer this genetic data obtained with primary cells into tests based on stable cell lines have proven surprisingly difficult (Lambrechts et al., 2009; Python et al., 2009). To date, no assay based on transcriptional changes has been submitted for (pre)validation, and the reasons for this could be (i) the difficulties in reproducing the gene expression changes in stable cell lines and (ii) the difficulties in fully standardizing RT-PCR based measurements.

We have proposed a pragmatic reporter cell-based approach based on the finding that the majority of the skin sensitizers induce the Nrf2-Keap1-ARE regulatory pathway (Natsch and Emter, 2008; Ade et al., 2009; Natsch, 2010; Vandebriel et al., 2010). The antioxidant response element (ARE) from the human AKR1C2 gene was inserted in front of a SV40 promotor and placed upstream of a luciferase gene. Stable insertion of the resulting construct in HaCaT keratinocytes resulted in the KeratinoSens reporter cell line. Induction of luciferase in this cell line can be used to screen for skin sensitizers. Since the Nrf2-Keap1-ARE regulatory pathway is involved in antioxidant response signalling, the ability to differentiate between sensitisation, irritation and antioxidant potential solely based on the ARE assay may have limitations but so far the assay has shown high predictivity. Indeed, the predictivity has been analyzed on a set of 67 reference test substances and an overall accuracy of 85.1% was determined. Due to this high predictivity and since it is based on the highly reproducible and technically simple luciferase-expression readout, this assay was considered a good candidate to be transferred to other laboratories and to enter (pre)validation. We report here the results of a detailed ring-study in five laboratories on a total of 28 reference substances.

2. Materials and methods

2.1. Test substances and study setup

The substances selected for this study cover (i) all the substances in the publication by Casati et al. (2009), which is largely overlapping with the list used by the Sens-it-iv consortium (Sens-it-iv, 2009) and (ii) all the substances in the draft performance standards for alternative endpoints in the LLNA published by ICCVAM (2008b). Diethyl phthalate was added as additional negative control from the list published by Sens-it-iv to make up the total number to 28 test substances. Supporting information 1 lists these substances, their commercial source and batch, CAS-number, and their sensitization potential as determined by the LLNA as given in the ICCVAM database (ICCVAM, 2008a) and in Basketter et al. (1999)).

This study-set was divided into two groups: A first set of 7 test substances was used to assess the transferability of the assay and for an initial assessment of the reproducibility (Phase I). The remaining 21 substances were sent to the laboratories blind-coded to assess the reproducibility and predictivity in detail (Phase II). The following substances were selected for Phase I: Three negatives (chlorobenzene, methyl salicylate, and sulfanilamide), three clear positives (DNCB, citral, and ethylene glycol dimethacrylate), and a borderline test substance according to the historical data published before (hexyl cinnamic aldehyde). The remaining 21 substances in Supporting information 1 were used for Phase II.

2.2. Cell line

The KeratinoSens cell line is derived from the human keratinocyte cell line HaCaT (Boukamp et al., 1988). It contains a stable insertion of a luciferase gene under the control of the ARE-element of the gene AKR1C2. The optimization of this cell line has been described in detail (Emter et al., 2010).

2.3. Test procedure and standard operating procedure (SOP)

All tests were run according to the previously published SOP (Emter et al., 2010). Briefly, cells were grown for 24 h in 96-well plates. The medium was then replaced with medium containing the test substance and a final level of 1% of the solvent, DMSO. Each test substance was tested at 12 twofold dilutions ranging from 0.98 to 2000 μM. Each 96-well test plate contained 7 serially diluted test substances, 6 wells with the solvent control, 1 well with no cells for background value and 5 wells with the positive control, cinnamic aldehyde, in five different concentrations. In each repetition, three parallel replicate plates were run with this same set-up and a fourth parallel plate was prepared for cytotoxicity determination. Cells were incubated for 48 h with the test substances, and then luciferase activity and cytotoxicity (with the MTT assay) were determined. This full procedure was repeated three times for each chemical, thus generating 9 luciferase induction data points and 3 MTT datapoints for each chemical at each concentration in each lab.

2.4. Controls and acceptance criteria

All the labs performed three repetitions consisting of three replicates on the Phase I substances and sent these data to the lead lab. Data quality was assessed by the lead lab whether they fulfil

the following criteria: (i) Variability in the 18 DMSO-control wells for each triplicate experiment is below 20% in all three repetitions (ii) dose–response curves are reproducible within the laboratory (i.e. increasing gene activation with increasing concentration up to the cytotoxic levels; EC1.5 and EC3 values which are no more then one well up and down in the dilution series from the average), and (iii) the positive control cinnamic aldehyde (contained in each test plate) gives a statistically significant induction above 1.5-fold below 64 μM in all three repetitions. Once this criteria were met by the three consecutive repetitions done by a particular lab, these data were taken as the final data of Phase I, and the individual labs were allowed to move into Phase II evaluation. Each lab then performed three repetitions consisting of three replicates on the Phase II substances and these data were directly used as the final data, and no data were rejected at this stage. This approach was chosen in order to avoid repeated testing and to gain experience on how robust the assay is and how narrowly the acceptance criteria should be defined in the future. More narrow criteria for the EC1.5 value of the positive control cinnamic aldehyde were initially also defined, but experiments were accepted even if these criteria were not met, again to gain experience on how narrow the criteria should be applied in the future. The results for positive and negative controls are all reported in the results section and in the Supporting information.

2.5. Modifications in the different laboratories for luciferase measurements

Each lab used their own quality of fetal calf serum and D-MEM medium but otherwise strictly adhered to the SOP. The only significant difference amongst the laboratories was the use of different approaches for luminescence readings, as not all laboratories were equipped with the same luminometer. The lead lab and lab 3 used the Glomax luminometer from Promega (Duebendorf, Switzerland), lab 1 used the infinity F500 (Tecan, Männedorf, Switzerland), lab 4 used the FLUOstar OPTIMA (BMG Labtech Inc., Cary, NC, USA) and lab 2 used the Orion II/MPL4 microplate luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). All laboratories used an injector and a flash substrate to inject the substrate to the lysed cells immediately before luciferase readings, with the exception of lab 2 which used the Perkin–Elmer Neolite Assay Kit, which is added simultaneously to all wells of the entire assay plate without the need of an injector.

2.6. Data analysis and statistical evaluation

For each chemical in each repetition and at each concentration, the gene induction compared to DMSO controls and the wells with statistically significant induction over the threshold of 1.5 (i.e. 50% enhanced gene activity) were determined. Furthermore the maximal fold-induction (I_{max}) and the EC1.5 value (concentration in μM for induction above the threshold, based on linear extrapolation as done in the LLNA) were calculated. The following prediction model was applied: A substances is rated as positive if the following three criteria are fulfilled (i) The EC1.5 value is below 1000 μM in all three repetitions or in at least 2 repetitions, (ii) at the lowest concentration with a gene induction above 1.5-fold (i.e. at the EC 1.5 determining value), the cellular viability is above 70% and (iii) there is an apparent overall dose–response for luciferase induction, which is similar between the repetitions.

To quantitatively compare the EC1.5 values and the IC50 values for cytotoxicity from the different laboratories, a logarithmic evaluation of the variability was made. The logarithmic values of the concentration data (EC1.5 and IC50) were calculated with the base 2. This is more intuitive as compared to base 10, since twofold dilutions were tested. Thus the Log_2-transformed values correlate to the number of wells in the plate to reach EC1.5/IC 50 values. Based on these Log_2-transformed values, the logarithmic standard deviations were calculated. These values were then re-transformed calculating the exponential function with base 2 (i.e. $2^{stdev(log\ values)}$), thereby rendering the geometrical standard deviation, which corresponds to a factor. (Numerical example: If the standard deviation of the Log_2 transformed values is 0.5, the geometric standard deviation is 1.414 or the square root of 2. The logarithmic 95.4% confidence interval then becomes ± 1 (i.e. twice the standard deviation) and the geometric (or re-transformed) 95.4% confidence interval is confined by a factor of 2. Thus in this specific case, the 95.4% confidence interval is covered by the concentration range one well in the microtiter plate up and down of the geometric mean.) There are two reasons for this approach: (i) the data approximate a log-normal distribution better than a normal distribution (data not shown) and therefore logarithmic calculations better describe the variability of the data and (ii) the geometric standard deviations are scale-independent and can be compared between substances of differing potency.

3. Results

3.1. Phase I – Transferability phase

No face-to-face training was required to transfer the method, and the SOP was found sufficiently detailed to perform the test in all the four external laboratories. There were no significant technical obstacles specific to the method. The key technical issues identified in the transfer phase were due to the different luminometer reading methods which initially yielded variable background readings and/or gradients over the assay plates in some labs. These issues are reviewed in the discussion section. Once they were solved, each lab performed three successive and successful repetitions and no further data needed to be discarded. All dose–response graphs for the 7 test substances tested in Phase I are presented in Supporting information 2. The results for DNCB and sulfanilamide are shown as examples in Figs. 1 and 2. Table 1 shows the numerical analysis of the data from phase I (I_{max}, EC1.5 and IC50 values and number of positive repetitions).

The positive test substances, DNCB, citral and ethylene glycol dimethacrylate, were positive in all 5 laboratories, and in all three repetitions. The three negative substances, chlorobenzene, methyl salicylate and sulfanilamide, were overall negative in all labs, with a few cases of a borderline induction in one of the three repetitions. The dose–response curves clearly confirm the positive rating for the three positive test substances and the negative rating for the three negatives (see Supporting information 2). The borderline test substance, hexyl cinnamic aldehyde, was consistently positive in two labs, negative in one lab, and gave a mixed result in two labs.

The EC1.5 values for DNCB and citral were similar in the four external labs and also close to the historical and new data of the lead lab. Also, for hexyl cinnamic aldehyde, the EC1.5 values in the positive repetitions show little variance (Table 1). However, a somewhat higher variation was observed for ethylene glycol dimethacrylate. The IC50 measure for cytotoxicity also proved to be reliable. For example, for DNCB the historical and the ring-study IC50 values of the lead lab were 8.2 and 10.1 μM, respectively, and the values from the four external labs were all between 6.6 and 12.5 μM.

3.2. Phase II – results for blind-coded substances

Once the laboratories had successfully tested the seven test substances of Phase I, they progressed to testing the 21 blind-coded substances of Phase II. These dose–response curves are all

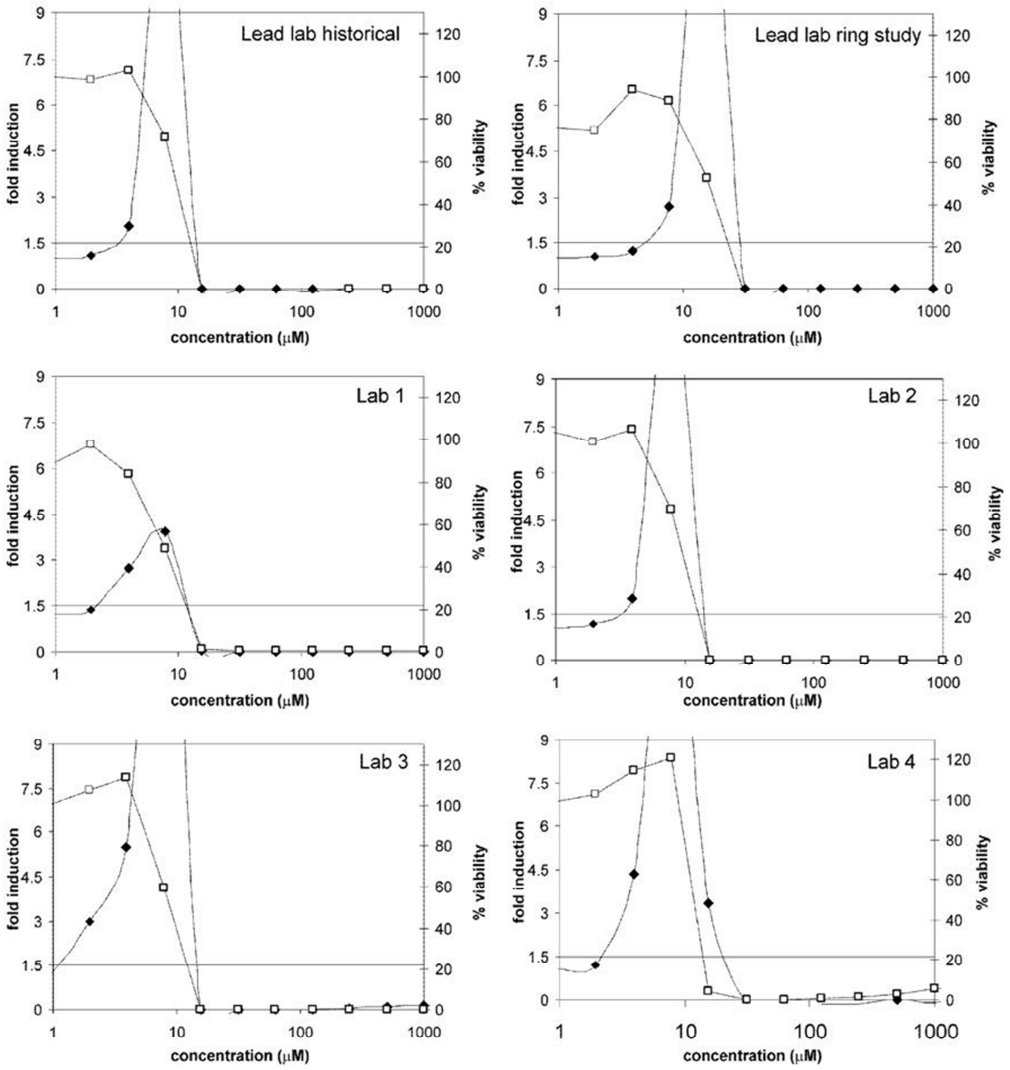

Fig. 1. Induction of luciferase activity (closed diamonds) and cellular viability (open squares) for DNCB in full dose–response analysis according to the SOP. Historical results of the lead lab and new data from the five labs in the transferability phase (Phase I). Graphs represent average results of 9 data points (3 independent trials with 3 replicates per trial).

presented in Supporting information 3, and the I_{max}, EC1.5 and IC50 values are summarized in Table 2. As illustrated by the dose–response graphs, similar overall dose–response results for these blind-coded test substances were obtained by the different labs. The biggest variation was observed in the dynamic range, i.e. the maximal gene induction I_{max} can vary significantly between the laboratories. However, the EC1.5, which had been found the key parameter to quantify the luciferase response (Natsch et al., 2009), appears more reproducible and is further analyzed quantitatively below.

Among the 15 sensitizers tested in Phase II, 11 were rated positive in all three repetitions in all five labs, and for 2 sensitizers there was one single negative repetition in one lab. For these 13 test substances the dose response curves are very clear. Phenyl benzoate is a false-negative in the historical data and this was confirmed with three negative repetitions in four labs, with one single positive repetition in one lab.

The only chemical which gave contradicting results in the between laboratory reproducibility (BLR) assessment of phase II is eugenol. In three labs, this test item was negative in 2 out of 3 repetitions and it was negative in the historical data, but rated positive in all repetitions in two labs with very reproducible dose–response curves. Among the 6 non-sensitizers, 4 were rated negative in all three repetitions in all the five labs. SLS was negative in four labs with 1–3 repetitions giving significant induction at cytotoxic concentrations only. There was one exception: in one lab significant luciferase induction was observed at the same concentration as in the other labs, but the cells were still fully viable in the parallel MTT plates at the inducing concentration. Diethyl phthalate was negative in four labs, but positive in one lab. However, the positive induction was paralleled by an increase of the MTT value to 170% (see Supporting information 3).

The data from both study phases were then further analyzed quantitatively to evaluate different aspects of the reproducibility,

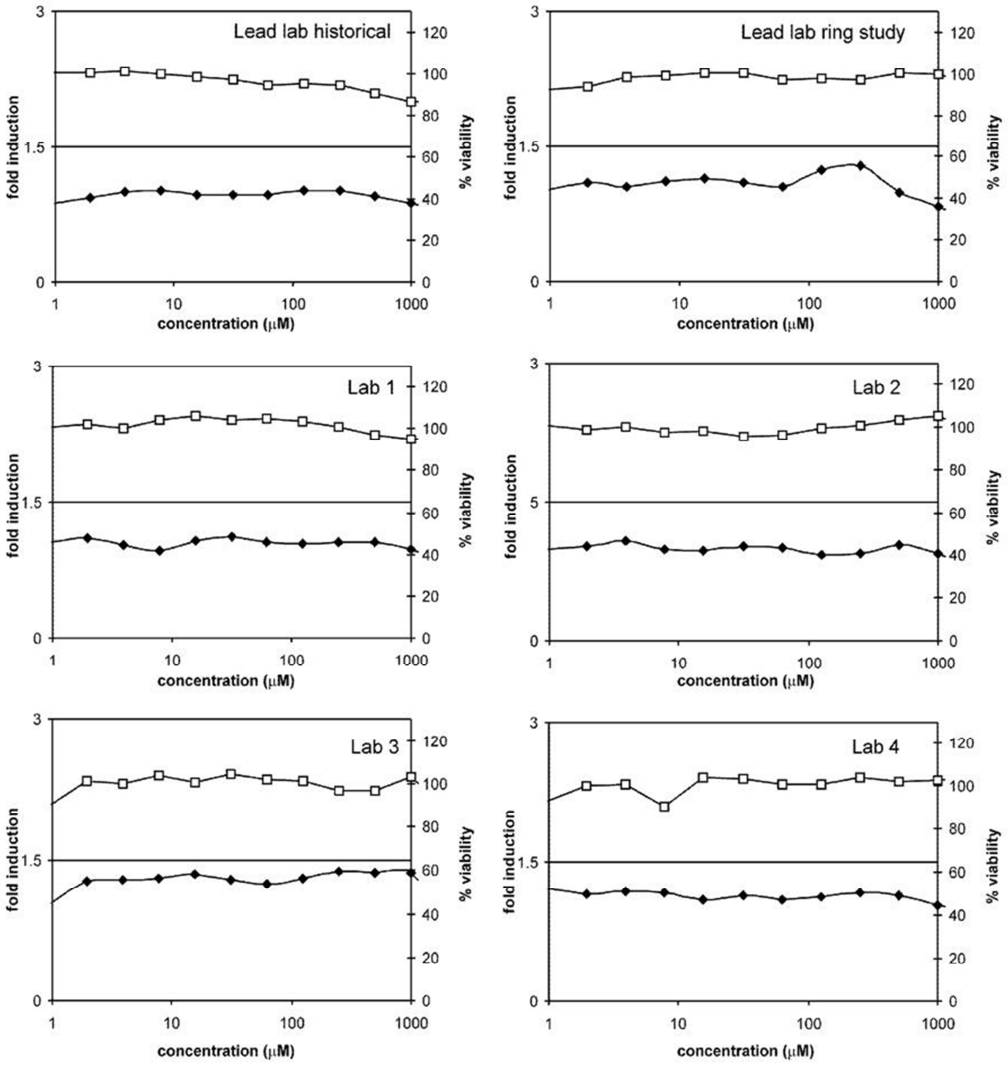

Fig. 2. Induction of luciferase activity (closed diamonds) and cellular viability (open squares) for sulfanilamide in full dose–response analysis according to the SOP. Historical results of the lead lab and new data from the five labs in the transferability phase.

and the data calculated below refer to results from both study phases.

3.3. Assessment of within laboratory reproducibility (WLR) of EC1.5 values

The lead lab had previously screened the set of test substances (Emter et al., 2010) and repeated this analysis within the ring-study. These data were first analyzed to determine the WLR of the EC1.5 values in the lead lab. For each chemical 5–7 repetitions were run in these two studies and these individual results are summarized in Supporting information 4. Based on these data, the geometric mean and the geometric standard deviation of the EC1.5 values were calculated as described in the methods section. The results are presented in Supporting information 5. The geometric standard deviation on the average for all positive chemicals of Phase I and Phase II is at 1.61, with one significant outlier (MCI). Without this outlier it is at 1.38. This is slightly below the square root of 2, indicating that, on the average, the 95.4% confidence interval for within laboratory variation of the EC1.5 value lies within one well up and down of the geometric mean in the dilution series (for details see the methods section).

A separate analysis of the WLR was then made in each of the five laboratories based on the three repetitions of the ring study. For each test substance and each laboratory the geometric standard deviation of the EC1.5 values was calculated (Table 3). The average of these geometric standard deviations for WLR was then calculated for each test substance (column WLR in Table 3). This value ranged between 1.1 and 1.8 for all of the test substances, with an average for all positive substances of 1.44. This is again close to the square root of 2. The average for all test substances within a lab is in the range between 1.27 and 1.69 for the different labs and generally close to 1.38 found above for the WLR in the

Table 1
Luciferase induction and cytotoxicity in the KeratinoSens assay for the seven chemicals of the transferability phase (Phase I).

		I_{max} (fold induction)[a]	EC 1.5 (μM)[b]	Positive repetitions[c]	IC 50 (μM)[d]
2,4-Dinitro-chlorobenzene	Lead_lab_historical	14.8	2.5	2 of 2	8.2
	Lead_lab	12.9	3.3	3 of 3	10.1
	Lab1	4.3	2.1	3 of 3	6.6
	Lab2	12.2	3.0	3 of 3	9.6
	Lab3	19.5	1.4	3 of 3	8.5
	Lab4	15.6	2.1	3 of 3	12.5
Citral	Lead_lab_historical	96.4	23.1	2 of 2	182.8
	Lead_lab	60.2	17.2	3 of 3	103.5
	Lab1	22.3	12.6	3 of 3	159.3
	Lab2	40.0	20.4	3 of 3	171.8
	Lab3	104.4	16.1	3 of 3	166.9
	Lab4	50.2	16.3	3 of 3	238.8
Ethylene glycol dimethacrylate	Lead_lab_historical	188.4	56.5	2 of 2	1655.8
	Lead_lab	176.9	81.5	3 of 3	909.1
	Lab1	25.8	112.2	3 of 3	879.9
	Lab2	102.1	54.6	3 of 3	871.5
	Lab3	363.0	10.6	3 of 3	963.2
	Lab4	204.9	42.0	3 of 3	1770.2
Hexyl cinnamic aldehyde	Lead_lab_historical	2.7	17.2	2 of 2	26.3
	Lead_lab	1.8	n.i.	1 of 3	30.9
	Lab1	1.4	n.i.	0 of 3	39.3
	Lab2	1.5	n.i.	1 of 3	31.1
	Lab3	4.2	23.5	3 of 3	62.7
	Lab4	5.3	17.2	3 of 3	90.9
Methyl salicylate	Lead_lab_historical	1.2	n.i.	0 of 2	>2000
	Lead_lab	1.4	n.i.	0 of 3	>2000
	Lab1	2.7	n.i.	1 of 3	>2000
	Lab2	1.5	n.i.	0 of 3	>2000
	Lab3	1.5	n.i.	1 of 3	>2000
	Lab4	1.5	n.i.	1 of 3	>2000
Chlorobenzene	Lead_lab_historical	1.2	n.i.	0 of 2	>2000
	Lead_lab	1.3	n.i.	0 of 3	>2000
	Lab1	1.5	n.i.	0 of 3	>2000
	Lab2	1.3	n.i.	0 of 3	>2000
	Lab3	1.3	n.i.	0 of 3	>2000
	Lab4	1.8	n.i.	1 of 3	>2000
Sulfanilamide	Lead_lab_historical	1.4	n.i.	0 of 2	>2000
	Lead_lab	1.1	n.i.	0 of 3	>2000
	Lab1	1.2	n.i.	0 of 3	>2000
	Lab2	1.1	n.i.	0 of 3	>2000
	Lab3	1.5	n.i.	1 of 3	>2000
	Lab4	1.3	n.i.	0 of 3	>2000

[a] Maximal fold-induction of luciferase activity in any of the 12 test concentrations, given are the averages of the three repetitions within each lab.
[b] Concentration in μM to reach 1.5-fold induction of gene activity, n.i. indicates no statistically significant induction above the threshold. Given are the geometric means of the three repetitions within each lab.
[c] Number of repetitions rating a chemical positive according to the prediction model (Significant induction > 1.5-fold below 1000 μM and at non-cytotoxic concentrations).
[d] Concentration in μM which reduces cellular viability by 50%. Given are the geometric means of the three repetitions within each lab.

Lead Lab. Therefore, on average a similar WLR for EC 1.5 values was found in all laboratories.

3.4. Assessment of between laboratory reproducibility (BLR) of EC 1.5 values

For each chemical the logarithmic average of the EC1.5 values per lab was calculated, and the standard deviation between the laboratories over these logarithmic averages was determined for each test substance. This value was then re-transformed to yield the geometric standard deviation of the EC1.5 between the laboratories (column BLR in Table 3). This parameter varied between 1.2 and 2.6 with an average of 1.64 (1.56 without the outlier MCI), indicating that the between laboratory variability is slightly higher as compared to the within laboratory variability. Still, for the majority of the substances it is below 1.41. Therefore, also for the BLR the 95.4% confidence interval of the EC1.5 value is, for most substances, within one well up and down from the geometric mean.

3.5. Assessment of WLR and BLR of IC50 values

The geometric means of the IC50 values for cytotoxicity of all test substances are listed in Tables 1 and 2, and in general congruent results within and between the labs were obtained. To quantify this, the within and between lab geometric standard deviations of the IC50 values were calculated (as done for the EC1.5 values in Table 3). These values could be calculated for those 18 test substances with a reduction of viability >50% at any of the test concentrations and they are listed in Table 4. The average of these geometric standard deviations within individual labs ranged between 1.09 and 1.81 for all of the test substances, with an average for all cytotoxic substances of 1.35, indicating that also for the WLR of the cytotoxicity values the 95.4% confidence interval is confined by a factor of less than 2. The BLR geometric standard deviations were between 1.08 and 1.94 for all test substances, with an average of 1.42. Thus, the variability of the IC50 values between the laboratories is even lower as compared to the variability of the EC1.5 values.

Table 2
Luciferase induction and cytotoxicity in the KeratinoSens assay for the 21 blind-coded chemicals of the reproducibility phase (Phase II).

		I_{max} (fold induction)[a]	EC 1.5 (μM)[b]	Positive repetitions[c]	IC 50 (μM)[d]
Non-sensitizers					
Lactic acid	Lead Lab	1.7	n.i.	0 of 3	>2000
	Lab1	1.3	n.i.	0 of 3	>2000
	Lab2	1.3	n.i.	0 of 3	>2000
	Lab3	1.2	n.i.	0 of 3	>2000
	Lab4	1.1	n.i.	0 of 3	>2000
Glycerol	Lead Lab	1.4	n.i.	0 of 3	>2000
	Lab1	1.2	n.i.	0 of 3	>2000
	Lab2	1.3	n.i.	0 of 3	>2000
	Lab3	1.2	n.i.	0 of 3	>2000
	Lab4	1.1	n.i.	0 of 3	>2000
Diethyl phthalate	Lead Lab	1.2	n.i.	0 of 3	>2000
	Lab1	1.2	n.i.	0 of 3	>2000
	Lab2	1.4	n.i.	1 of 3	>2000
	Lab3	1.7	n.i.	0 of 3	>2000
	Lab4	1.9	582.7	2 of 3	>2000
Isopropanol	Lead Lab	1.3	n.i.	0 of 3	>2000
	Lab1	1.3	n.i.	0 of 3	>2000
	Lab2	1.4	n.i.	0 of 3	>2000
	Lab3	1.5	n.i.	0 of 3	>2000
	Lab4	1.1	n.i.	0 of 3	>2000
Salicylic acid	Lead Lab	1.1	n.i.	0 of 3	>2000
	Lab1	1.4	n.i.	0 of 3	>2000
	Lab2	1.3	n.i.	0 of 3	>2000
	Lab3	1.3	n.i.	0 of 3	>2000
	Lab4	1.3	n.i.	0 of 3	>2000
Sodium lauryl sulphate	Lead Lab	5.9	35.3	3 at cytotox	57.0
	Lab1	1.4	n.i.	1 at cytotox	74.5
	Lab2	5.1	n.i.	1 at cytotox	54.5
	Lab3	2.7	n.i.	1 at cytotox	46.2
	Lab4	8.1	33.9	3 of 3	91.1
Sensitizers					
4-Phenylenediamine	Lead Lab	45.2	9.9	3 of 3	400.7
	Lab1	23.2	10.1	3 of 3	396.4
	Lab2	19.4	8.4	3 of 3	577.2
	Lab3	39.0	6.0	3 of 3	648.9
	Lab4	20.0	10.1	3 of 3	427.0
Cinnamic aldehyde	Lead Lab	22.4	14.3	3 of 3	164.1
	Lab1	9.8	14.8	3 of 3	172.6
	Lab2	32.4	6.0	3 of 3	132.6
	Lab3	44.7	7.5	3 of 3	348.9
	Lab4	22.2	6.5	3 of 3	113.1
4-Nitrobenzylbromide	Lead Lab	7.0	1.4	3 of 3	7.2
	Lab1	4.7	<0.98	3 of 3	5.7
	Lab2	13.6	1.4	3 of 3	13.7
	Lab3	14.0	<0.98	3 of 3	10.8
	Lab4	9.7	<0.98	3 of 3	8.3
Metol	Lead Lab	5.9	8.7	3 of 3	52.7
	Lab1	4.5	3.2	3 of 3	31.8
	Lab2	9.4	5.7	3 of 3	53.6
	Lab3	12.2	6.6	3 of 3	137.0
	Lab4	6.4	3.5	3 of 3	24.0
Isoeugenol	Lead Lab	13.2	18.4	3 of 3	550.9
	Lab1	9.5	23.3	3 of 3	560.6
	Lab2	23.2	10.4	3 of 3	997.5
	Lab3	56.8	4.5	3 of 3	791.6
	Lab4	14.4	22.9	3 of 3	515.7
Eugenol	Lead Lab	1.4	n.i.	1 of 3	1363.2
	Lab1	1.4	n.i.	1 of 3	1281.4
	Lab2	2.5	309.1	3 of 3	1496.6
	Lab3	2.5	231.2	3 of 3	1487.1
	Lab4	1.7	n.i.	1 of 3	1538.0
Oxazolone	Lead Lab	24.3	191.2	3 of 3	1667.8
	Lab1	6.5	185.2	3 of 3	1761.0
	Lab2	19.3	240.8	3 of 3	>2000
	Lab3	46.3	152.9	3 of 3	>2000
	Lab4	6.4	84.3	3 of 3	777.9

(*continued on next page*)

Table 2 (*continued*)

		I_{max} (fold induction)[a]	EC 1.5 (μM)[b]	Positive repetitions[c]	IC 50 (μM)[d]
2-Mercapto-benzothiazole	Lead Lab	24.5	44.3	3 of 3	983.4
	Lab1	4.9	108.0	3 of 3	1099.6
	Lab2	54.2	54.4	3 of 3	1576.6
	Lab3	64.1	65.5	3 of 3	1656.1
	Lab4	21.5	226.9	3 of 3	1429.1
(5-Chloro)-methyl-isothiazolinone	Lead Lab	7.6	7.6	3 of 3	32.6
	Lab1	4.9	1.0	3 of 3	8.9
	Lab2	4.8	4.2	3 of 3	25.6
	Lab3	13.5	0.8	3 of 3	14.5
	Lab4	7.8	2.8	3 of 3	12.8
Imidazolidinyl urea	Lead Lab	1.8	50.0	2 of 3	92.7
	Lab1	3.6	35.9	3 of 3	80.5
	Lab2	5.1	29.9	3 of 3	125.2
	Lab3	11.5	31.3	3 of 3	103.0
	Lab4	9.2	32.2	3 of 3	94.7
Methyldibromo glutaronitrile	Lead Lab	5.2	12.4	3 of 3	34.4
	Lab1	2.1	7.2	3 of 3	24.7
	Lab2	6.1	10.9	3 of 3	53.5
	Lab3	7.8	7.4	3 of 3	43.5
	Lab4	3.3	6.7	3 of 3	31.8
Glyoxal	Lead Lab	67.1	134.1	3 of 3	610.5
	Lab1	14.5	77.0	3 of 3	523.2
	Lab2	58.3	120.0	3 of 3	721.9
	Lab3	195.0	95.6	3 of 3	>800
	Lab4	69.7	169.1	3 of 3	700.4
Cinnamyl alcohol	Lead Lab	10.8	104.1	3 of 3	1705.3
	Lab1	1.7	106.6	2 of 3	1726.3
	Lab2	17.1	86.3	3 of 3	1827.8
	Lab3	9.1	130.9	3 of 3	>2000
	Lab4	8.6	118.2	3 of 3	>2000
Tetramethyl-thiuramdisulfide	Lead Lab	22.3	<0.98	3 of 3	23.8
	Lab1	8.1	<0.98	3 of 3	26.8
	Lab2	17.7	4.1	3 of 3	53.7
	Lab3	67.9	1.9	3 of 3	36.6
	Lab4	9.3	<0.98	3 of 3	11.8
Phenyl benzoate	Lead Lab	1.0	n.i.	0 of 3	185.2
	Lab1	1.2	n.i.	0 of 3	263.3
	Lab2	1.2	n.i.	0 of 3	309.6
	Lab3	1.2	n.i.	0 of 3	239.9
	Lab4	2.4	n.i.	1 of 3	808.6

[a] Maximal fold-induction of luciferase activity in any of the 12 test concentrations, given are the averages of the three repetitions within each lab.

[b] Concentration in μM to reach 1.5-fold induction of gene activity, n.i. indicates no statistically significant induction above the threshold. Given are the geometric means of the three repetitions within each lab.

[c] Number of repetitions rating a chemical positive according to the prediction model (Significant induction > 1.5-fold below 1000 μM and at non-cytotoxic concentrations).

[d] Concentration in μM which reduces cellular viability by 50%. Given are the geometric means of the three repetitions within each lab.

3.6. Assessment of BLR of the predictive capacity (PC)

In Fig. 3, the positive (red and orange) and negative (dark and faint green) ratings for all the 28 substances are summarized, and the Cooper statistics calculated. The accuracy was between 85.7 and 96.4% in the different laboratories. The main reason for the difference in accuracy between the laboratories is the different rating of the borderline chemical hexyl cinnamic aldehyde, and the different results obtained for eugenol. The outliers for diethyl phthalate and SLS in lab 4 affected the Cooper statistics somewhat for this lab.

3.7. Quality control values

Finally, the controls included in all assay plates (DMSO controls and positive control cinnamic aldehyde) were compared across laboratories (see Supporting information 6). Once the test was set up and running in the different laboratories, the performance criteria for a variability of <20% in the DMSO-control wells was fulfilled in 54 of the 60 runs, and it was between 20% and 26% in the remaining 6 runs. The average variability for all runs was 13.3%. The performance criteria that cinnamic aldehyde was significantly positive (EC1.5 < 64 μM) was fulfilled in all of the 60 runs. The quantitative performance criteria for the induction by cinnamic aldehyde (a) Induction at 64 μM between 2 and 8-fold was fulfilled in 51 of the 60 runs, and (b) EC1.5 between 7 μM and 30 μM was fulfilled in 51 of the 60 runs. Generally these results indicate that cinnamic aldehyde is a very robust positive control to verify that the test is working and it is reliably positive in all the runs in all the labs. However, the quantitative variability for the EC1.5 value for cinnamic aldehyde when tested as positive reference was clearly higher as compared to some other substances studied here. Interestingly, this variability was lower when cinnamic aldehyde was tested as a blind coded chemical (see Table 2). The reason for this is currently unknown.

4. Discussion

The results of this study indicate that the KeratinoSens assay, based on a reporter gene read-out, is easily transferable between

Table 3
Geometric standard deviations of the EC1.5 values for the consistently positive[a] chemicals.

	Geometric standard deviations						
	Lead lab[b]	Lab 1	Lab 2	Lab 3	Lab 4	WLR[c]	BLR[d]
Metol	1.62	1.71	2.23	1.17	2.24	1.74	1.53
(5-Chloro)-methylisothiazolinone	1.98	2.33	1.62	1.75	1.44	1.80	2.56
Imidazolidinyl urea	1.00	1.08	1.52	1.07	1.03	1.13	1.23
Oxazolone	1.03	1.91	1.76	1.20	1.58	1.46	1.49
4-Phenylenediamine	1.13	1.29	1.77	1.07	2.88	1.52	1.25
Cinnamic aldehyde	1.10	1.06	1.32	1.32	2.01	1.33	1.55
Isoeugenol	1.57	1.82	1.68	2.07	1.50	1.71	2.02
2-Mercaptobenzothiazole	1.25	1.62	3.09	1.05	1.38	1.55	1.92
Cinnamyl alcohol	1.17	2.28	1.26	1.27	1.09	1.36	1.17
Glyoxal	1.04	2.70	1.05	1.17	1.34	1.36	1.35
Methyldibromo glutaronitrile	1.36	1.51	1.41	1.13	1.16	1.30	1.32
Citral	1.12	1.73	1.61	1.90	1.09	1.45	1.19
Ethylene glycol dimethacrylate	1.19	1.22	1.10	1.42	1.16	1.21	2.49
2,4-Dinitrochlorobenzene	1.29	1.34	1.27	1.43	1.02	1.26	1.40
Average	1.27	1.69	1.62	1.36	1.49	1.44	1.60

[a] Hexyl cinnamic aldehyde and eugenol, showed induction in some labs only, thus not evaluated; tetramethylthiuramdisulfide and 4-nitrobenzyl-bromide, data contain several EC1.5 values <0.98, thus could not be used for this statistical analysis. However also for these four test substances the observed EC1.5 values were reproducible as shown in Tables 1 and 2.
[b] For each lab the geometric standard deviation of the three repetitions was calculated.
[c] Indicates the average geometric standard deviation within the different labs.
[d] Indicates the geometric standard deviation of the logarithmic averages of each lab.

Table 4
Geometric standard deviations of the IC50 values for the chemicals which were cytotoxic at the tested doses in the majority of the labs.

	Geometric standard deviations						
	Lead lab[a]	Lab 1	Lab 2	Lab 3	Lab 4	WLR[b]	BLR[c]
2,4-Dinitrochlorobenzene	1.10	1.73	1.24	1.30	1.02	1.28	1.27
4-Phenylenediamine	1.05	1.01	1.40	1.40	2.14	1.40	1.26
Cinnamic aldehyde	1.20	1.56	1.18	1.49	1.06	1.30	1.54
4-nitrobenzylbromide	1.41	2.83	1.73	1.39	1.70	1.81	1.41
4-Methylaminophenol sulphate (METOL)	1.68	1.96	1.02	1.27	2.34	1.65	1.94
Isoeugenol	1.17	2.03	1.44	1.13	1.37	1.43	1.33
Eugenol	1.04	1.33	1.01	1.03	1.03	1.09	1.08
2-Mercaptobenzothiazole	1.08	1.36	1.06	1.10	1.27	1.17	1.26
(5-chloro)-Methylisothiazolinone	2.38	2.07	1.01	1.51	1.57	1.71	1.70
Sodium lauryl sulphate	1.10	1.30	1.46	1.04	1.05	1.19	1.31
Imidazolidinyl urea	1.02	1.28	1.48	1.05	1.01	1.17	1.18
Methyldibromo glutaronitrile	1.35	1.28	1.08	1.06	1.28	1.21	1.34
Glyoxal	1.03	1.38	1.08	n.c. [d]	1.01	1.12	1.16
tetramethylthiuramdisulfide	1.07	1.66	1.02	1.32	1.19	1.25	1.75
Phenyl benzoate	1.02	1.56	2.25	1.45	2.06	1.67	1.76
Citral	1.04	1.27	1.43	1.07	1.05	1.17	1.35
Ethylene glycol dimethacrylate	1.06	1.68	1.04	1.37	n.c.	1.29	1.35
Hexyl cinnamic aldehyde	1.19	1.60	1.45	1.44	1.08	1.35	1.60
Averages	1.22	1.60	1.30	1.26	1.36	1.35	1.42

[a] For each lab the geometric standard deviation of the three repetitions was calculated.
[b] Indicates the average geometric standard deviation within the different labs.
[c] Indicates the geometric standard deviation of the logarithmic averages of each lab.
[d] n.c. not cytotoxic at maximal test concentration.

labs. In general, the predictive capacity is similar between labs, and more importantly, also the quantitative dose–response data were reproduced in the different laboratories. The fact that the between laboratory variability for EC1.5 values was only slightly above the within laboratory variability indicates that transfer of the assay to other labs does not affect the results significantly.

4.1. Hurdles for transferability

There were some minor hurdles in the transferability phase, but these were not specific to the assay but rather specific to the use of any luciferase based assay. The key issues identified in the transfer phase were due to three reasons (a) The use of differing luminometers (b) the use of a glow-substrate instead of a flash-substrate for the luciferase measurements. Glow-substrates emit low levels of light for prolonged periods, which contributes to lower sensitivity and thus higher variability. Longer reading times for plates with the glow-substrate then caused a gradient over the assay plate in one lab. This lab then switched to a flash-substrate. Nevertheless, one laboratory did successfully use the glow-approach. (c) The use of assay plates which do not properly fit the height of the luminometer, or allow light to scatter into adjacent wells. Especially when using the glow-approach, the initial plates used allowed light scattering into the adjacent rows, and thus initial false-positive results in two labs. To ensure accurate determinations of EC1.5 values (i) a high sensitivity, (ii) a low variability (and thus a stable background) and (iii) no gradient over the plate were found to be absolutely critical. A training experiment was designed based on these experiences and this may be used in future transfers to ensure the above three parameters are met. Once these hurdles (in

	Study phase	Positive with EC 1.5 up to 1000 µM					
		Lead Lab hist.	Lead lab	Lab 1	Lab 2	Lab 3	Lab 4
Sensitizers							
Hexyl cinnamic aldehyde	MT	2 of 2	1 of 3	0 of 3	1 of 3	3 of 3	3 of 3
Citral	MT	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Ethylene glycol dimethacrylate	MT	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
2,4-Dinitrochlorobenzene	MT	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
4-Methylaminophenol sulphate	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
(5-Chloro)-methylisothiazolinone	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Phenyl benzoate	BC	1 of 4	0 of 3	0 of 3	0 of 3	0 of 3	1 of 3
Imidazolidinyl urea	BC	3 of 4	2 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Oxazolone	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
4-Phenylenediamine	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Cinnamic aldehyde	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Isoeugenol	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Tetramethylthiuramdisulfide	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
2-Mercaptobenzothiazole	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Eugenol	BC	0 of 4	1 of 3	1 of 3	3 of 3	3 of 3	1 of 3
Cinnamyl alcohol	BC	4 of 4	3 of 3	2 of 3	3 of 3	3 of 3	3 of 3
Glyoxal	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
4-Nitrobenzylbromide	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Methyldibromo glutaronitrile	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Non-sensitizers							
Isopropanol	BC	0 of 2	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3
Methyl salicylate	MT	0 of 2	0 of 3	1 of 3	0 of 3	1 of 3	1 of 3
Chlorobenzene	MT	0 of 2	0 of 3	0 of 3	0 of 3	0 of 3	1 of 3
Sulfanilamide	MT	0 of 2	0 of 3	0 of 3	0 of 3	1 of 3	0 of 3
Salicylic acid	BC	0 of 2	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3
Sodium lauryl sulfate	BC	0 of 2	3 at cyto-tox. conc.	1 at cyto-tox. conc.	1 at cyto-tox. conc.	1 at cyto-tox. conc.	3 of 3
Lactic acid	BC	1 of 4	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3
Glycerol	BC	0 of 4	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3
Diethyl phthalate	BC	0 of 2	0 of 3	0 of 3	1 of 3	0 of 3	2 of 3
Cooper statistics							
correct positive		17	16	16	17	18	17
correct negative		9	9	9	9	9	7
false positive		0	0	0	0	0	2
false negative		2	3	3	2	1	2
n		28	28	28	28	28	28
Sensitivity (%)		89.5	84.2	84.2	89.5	94.7	89.5
Specificity (%)		100.0	100.0	100.0	100.0	100.0	77.8
Accuracy (%)		92.9	89.3	89.3	92.9	96.4	85.7

Fig. 3. The predictive capacity in the different labs. If 2 of 3 reps are positive and overall dose response is given in all reps, compound is considered positive (red and orange). If only one rep is positive and dose response is not evident compound is considered negative (dark and faint green). The induction at cytotoxic concentrations for SDS was not considered positive. MT: method transfer/phase I data (7 test substances), BC: blind study/phase II data (21 test substances). Detailed results are presented in the dose–response curves in Supporting information 2 and 3 and in Tables 1 and 2. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

the physical, not the biological part of the experiment) were overcome, the transferability was found to be very good, and the biological system did not pose any challenges in the lab-to-lab transfer.

4.2. Outliers

Not too surprisingly in an extended study with 5 × 28 individual test substance assessments, a few outliers were recorded. The borderline chemical hexyl cinnamic aldehyde gave mixed results among labs, but was a known borderline test item in the historical data and it varied between historical and new data in the lead lab also. Thus in this case, the variability between the labs was reproducing a variability already observed within the lead lab. Hexyl cinnamic aldehyde becomes positive very close to the cytotoxic level, and the IC50 value for this test item was higher in the labs reporting a consistently positive result. The result for eugenol, a reported weak sensitizer, was more surprising. This chemical was positive in the AREc32 assay (Natsch and Emter, 2008) and positive in two testing labs, but negative in three labs including the lead lab. We had observed that HaCaT keratinocytes and EpiDerm™ skin models convert eugenol into a metabolite of high molecular weight (our unpublished observation), and efficient detoxification could be the reason for a lack of gene induction and the low cytotoxicity for eugenol. However, this does not explain the differing results in the participating labs. For SLS, our observation that the luciferase expression is induced at a very narrow range of cytotoxic concentrations was reproduced in several labs. This phenomenon had been previously illustrated by a detailed dose–response analysis (Emter et al., 2010). Still, there was an outlier in one lab with a positive induction at a concentration which was rated as non-cytotoxic in the parallel MTT-plate. Comparing the data of the different labs, the outlier appears rather at the level of the IC50 value, and not at the level of the EC1.5 value. This discrepancy could be due to the fact that cytotoxicity is not measured directly within the assay plates, and a possible improvement would be to include a cytotoxicity measure directly in the assay plates prior to luciferase readings as demonstrated by Uibel et al. (2010). Finally, one lab found a positive induction for diethyl phthalate which was paralleled by a strong increase in the MTT values. This might indicate that cell numbers increased, and that the luciferase production per cell remained constant. The reason for this effect is unknown.

4.3. Prediction model

Two changes were made to the prediction model, which had already been anticipated in our previous publication (Emter et al., 2010): (i) Test substances are rated positive if EC1.5 is below 1000 μM (previously 2000 μM), since occasional false-positive results were recorded at 2000 μM and (ii) only if the viability at the EC1.5-determining value is >70%, the result is considered positive. The reasons for these modifications had been discussed (Emter et al., 2010).

4.4. Cytotoxicity of the test molecules

The data in Tables 1 and 2 indicate that with the exception of SLS all tested non-sensitizers have very low cytotoxicity. One may suspect, based on this dataset, that more cytotoxic substances do activate the Nrf2 pathway, and that the difference between sensitizers and non-sensitizers is largely based on their cytotoxic properties. However, this is only due to this particular test set of non-sensitizers which was selected based on published lists and which consists of molecules of low cytotoxicity. We had screened large numbers of novel molecules in our discovery process, and many non-reactive but cytotoxic substances were identified, having IC50 values far below 2000 μM with no significant ARE-dependent luciferase activation. Thus, luciferase induction does not correlate to cytotoxicity as might be suspected based on the present data (our unpublished data).

4.5. Quality controls

In Phase II, all the experiments were accepted, even if slightly outside of the targeted performance criteria. With this approach, analysis of the data now indicates how sensitive the assay is relative to these performance criteria. This will allow an eventual redefining of the performance criteria for full validation studies, whereas if the performance criteria would have been strictly applied in the ring-study, it would not have been possible to determine how narrow the criteria need to be in order to obtain useful and reproducible results. The results indicated that the dose–response curves in a run with control variability of 8.4% are clearly smoother as compared to the data in a parallel run with 26.8% control variability (i.e. the maximal variability observed), which indicates that one should in the future strictly adhere to the 20% variability criterion. On the other hand, the data from runs with an EC1.5 value for the positive control cinnamic aldehyde outside of the target range were still of very good quality and this performance criterion may by defined less stringent based on the current data.

5. Conclusions

Reporter gene assays have widely been used to screen for hormone-active substances, and the corresponding tests had been assessed for transferability (van der Burg et al., 2010). This study is the first inter-laboratory study on gene expression changes induced by skin sensitizers in a stable cell line. The assay was reproducible between the laboratories for 26 out of the 28 chemicals and an overall accuracy for these 28 chemicals between 85.4 and 96.7% was reported from the five labs. More importantly, also the dose–response curves and the quantitative parameters (concentration for significant gene induction and IC50 values for cytotoxicity) were reproducible, and the between laboratory variability was only slightly higher as compared to the within laboratory variability. This high reproducibility of the results from the KeratinoSens assay in this extensive study with many substances and five participating labs encourages us to progress to official (pre)validation.

Supporting information

Supporting information 1 gives details on the test chemicals.

Supporting information 2 gives all the dose response curves in all the labs for Phase I.

Supporting information 3 gives all the dose response curves in all the labs for Phase II.

Supporting information 4 gives the detailed results on the WLR in the lead lab.

Supporting information 5 gives the statistical analysis of the WLR in the lead lab.

Supporting information 6 gives the results for the control values in all the labs and all the repetitions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tiv.2010.12.014.

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5 VALIDATION STUDY OF A BATTERY OF FOUR TEST SYSTEMS

5.1 INTRA-LABORATORY VALIDATION OF FOUR *IN VITRO* ASSAYS FOR THE PREDICTION OF THE SKIN SENSITIZING POTENTIAL OF CHEMICALS

Bauch C.; Kolle S. N.; Fabian E.; Pachel C.; Ramirez, T.; Wiench B.; Wruck, C.J.; van Ravenzwaay, B; Landsiedel, R.: *Intralaboratory validation of four in vitro assays for the prediction of the skin sensitizing potential of chemicals*; **Toxicology in vitro 25 (6)** SI p. 1162-1168; 2011, DOI: 10.1016/j.tiv.2011.05.030

This chapter describes an in-house validation study published in Toxicology In Vitro. The aim of this study was the performance of four *in vitro* assays with 23 chemicals of known sensitizing potential in order to directly compare these test systems and to spot differences in their capability to predict chemicals as sensitizers. The test chemicals were 2,4,6-trinitrobenzene and the 22 performance standards of the LLNA according the OECD guideline 429. Although this list of chemicals represents a small selection, it comprises a variety of non-sensitizers and sensitizers of various potencies as well as pro- and pre-haptens and metals. Performance of the same test substances with different assays allows a direct comparison and shows whether an assay is deficient in prediction capacity and if combination of assays into an ITS may compensate such underestimations. The four assays chosen for this study were the *in chemico* prediction of protein reactivity using the DPRA assay, activation of the Keap1-Nrf2-signaling pathway using the previous described KeratinoSens assay, and the detection of dendritic cell activation using dendritic cell-like cell lines THP-1 in the h-CLAT and U-937 in the MUSST.

The cell-free *in chemico* assay DPRA (described in Chapter 2.3.2 *in chemico* assays) was used to assess the potential of chemicals to react with peptides and proteins. Therefore both model peptides were incubated separately with 100 mM test substance at room temperature for 24 h. The mixture was then analyzed using a HPLC-UV, and free, non-reacted peptides were detected at 220 nm. If test a substance formed stable conjugates with the peptides the area under the curve was reduced compared to the peak of non-treated peptides, an effect called peptide depletion. In this work, a substance was classified as skin sensitizer if the averaged peptide depletion of cysteine and lysine peptide was greater than 6.376%, adopted from the decision tree by Gerberick and co-workers (Gerberick *et al.* 2007). The decision tree allows an estimation of the sensitizing potency but was neglected due to the lack of such prediction models of KeratinoSens, MUSST and h-CLAT. The results of tested chemicals showed that the DPRA predicted 21 out of 23 substances correctly.

The KeratinoSens assay, performed as described above (see chapter 4), predicted 20 out of 23 substances correctly. No substance was predicted as false positive.

The DC activation assay h-CLAT measures the activation of the DC like cell line THP-1, a human myeloid cell line. The readout is the up-regulation of CD86 and CD54, two cell surface makers of DC known to be up-regulated during the process of DC maturation. Treatment with test substance was performed over 24 h and cell surface expression of CD86 and CD54 were measured using antibody staining and flow cytometric analyses. Comparison to untreated cells indicated the changes in expression, and substances were rated as skin sensitizers if CD86 expression was greater than 150% and/or CD54 levels were greater than 200%. Results of the h-CLAT yielded a correct prediction for 20 out of 23 substances.

The counterpart to the h-CLAT is the DC activation assay MUSST which reflects the activation of the cell line U-937, a human monocytic cell line. Main differences to the h-CLAT, apart from the cell line, are the use of only CD86 as maturation marker and the exposure of cells with the substance for 48 h. Chemicals were rated as skin sensitizers if the expression of CD86 was greater than a 1.2-fold induction compared to control cells. Results of the MUSST showed a correct prediction of 20 out of 23 substances, whereas no false positive prediction occurred.

Calculation of the Cooper statistics compared to human patch test data from literature revealed sensitivities of 81 % for KeratinoSens, h-CLAT and MUSST and 93 % for DPRA. h-CLAT and DPRA exhibited specificities of 86 % and KeratinoSens and MUSST had a 100 % sensitivity. Overall, the validated *in vitro* assays had an accuracy to predict sensitizing potential of chemicals with 83 % (h-CLAT), 87 % (MUSST and KeratinoSens) and 91 % (DPRA). As the LLNA is the current prediction model for skin sensitization and is referred to as the gold standard, results were compared to literature data from the LLNA. Nickel chloride is predicted as non-sensitizer and SDS as sensitizer in the LLNA. Including these corrections the Cooper statistics changed to sensitivities of 75 % (MUSST), 81 % (KeratinoSens and h-CLAT) and 93 % (DPRA) whereas specificities ranged from 86 % (MUSST, h-CLAT, DPRA) and 100 % (KeratinoSens). The overall accuracies to predict sensitizing potential of chemicals were 78 % (MUSST), 83 % (h-CLAT), 87% (KeratinoSens) and 91 % (DPRA).

This study showed the successful validation of four different alternative, animal-free methods with the use of LLNA performance standards according the OCED guideline 429. The predictivities of each single assay were relatively high with accuracies above 83%. A stricter definition of the applicability domain leads to the exclusion of certain substances, e.g. pro-haptens and might lead to higher prediction but might also conceal some weak points of the assay.

The validation of KeratinoSens for this work presented was part of my PhD project. Assay performance and data evaluations were solely performed by myself. I have contributed to experiments for MUSST and h-CLAT performance and overtook the data evaluation of both assays. The data used from the DPRA assay were already existing and only data evaluation for this work had to be done. The arrangement of results and writing of the manuscript was also my duty.

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Intralaboratory validation of four *in vitro* assays for the prediction of the skin sensitizing potential of chemicals

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ABSTRACT

Allergic contact dermatitis is induced by repeated skin contact with an allergen. Assessment of the skin sensitizing potential of chemicals, agrochemicals, and especially cosmetic ingredients is currently performed with the use of animals. Animal welfare and EU legislation demand animal-free alternatives reflected in a testing and marketing ban for cosmetic ingredients beginning in 2013. The underlying mechanisms of induction and elicitation of skin sensitization are complex and a chemical needs to comply several properties being skin sensitizing. To account for the multitude of events in the induction of skin sensitization an *in vitro* test system will consist of a battery of various tests.

Currently, we performed intralaboratory validations of four assays addressing three different events during induction of skin sensitization. (1) The Direct Peptide Reactivity Assay (DPRA) according to Gerberick and co-workers (Gerberick et al., 2004) using synthetic peptides and HPLC analysis. (2) Two dendritic cell activation assays based on the dendritic cell like cell lines U-937 and THP-1 and flow cytometric detection of the maturation markers CD54 and/or CD86 (Ashikaga et al., 2006; Python et al., 2007; Sakaguchi et al., 2006). (3) Antioxidant response element (ARE)-dependent gene activity in a HaCaT reporter gene cell line (Emter et al., 2010). We present the results of our intralaboratory validation of these assays with 23 substances of known sensitizing potential. The sensitivity, specificity, and accuracy of the individual tests were obtained by comparison to human epidemiological data as well as to data from animal tests such as the local lymph node assay.

1. Introduction

Allergic contact dermatitis (ACD) is a common skin disease with a significant social and economic impact. ACD is induced by repeated skin contact with an allergen (hapten, pro-hapten or complete allergen). The assessment of the skin sensitizing potential of haptens like industrial chemicals, agrochemicals, and cosmetic ingredients is crucial to define their safe handling and use. Up to now, animal based methods using guinea pigs or mice are used to identify skin sensitizing potential (ICCVAM, 2008, 2009; OECD, 2010). Public concerning regarding animal welfare, which is also reflected in current legislation such as the 7th Amendment to the Cosmetics Directive (Council Directive 76/768/EEC, 1976) as well as REACH (Council Directive 1907/2006/EEC, 2006), demands animal free alternative methods. Especially for cosmetics and cosmetic ingredients testing with animals is restricted since 2009. The marketing ban for cosmetic with ingredients tested for skin sensitization in animals starts in March 2013. Currently there is no validated and accepted animal free alternative available for skin sensitization testing. The mechanisms of induction and elicitation of skin sensitization are complex. Briefly, the contact allergen needs to penetrate the skin and to be either reactive towards endogenous proteins itself or – as pro-hapten – which has to be metabolically activated in the skin. Subsequently, the elaboration of inflammatory responses by keratinocytes occurs via the secretion of inflammatory cytokines like IL-1α or IL-18. Following, the activation of dendritic cells (DC), the up-regulation of co-stimulatory cell surface markers, the secretion of cytokines, and

Abbreviations: ACD, allergic contact dermatitis; ARE, antioxidant response element; DC, dendritic cells; DMSO, dimethyl sulfoxide; DNCB, 1-chloro-2,4-dinitrobenzene; DPRA, direct peptide reactivity assay; FBS, fetal bovine serum; h-CLAT, human Cell Line Activation Test; Kathon CG, mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one; LLNA, local lymph node assay; MUSST, myeloid U-937 skin sensitization test; Nrf2, nuclear factor-erythroid 2- related factor 2; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

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migration of DC into lymph nodes takes place. These mature DC interact in the local lymph node with naive T cells and prime them to proliferate and differentiate into specific T cells (Kimber et al., 2002; Ryan et al., 2007). Due to the complexity of the underlying mechanisms, the interaction of different cell types like dendritic cells, keratinocytes and fibroblast a single *in vitro* test is likely not able to cover the all steps of skin sensitization (dos Santos et al., 2009; Corsini et al., 2009). Hence, an *in vitro* test system will consist of a battery of various tests covering several steps of the described process. The aim of this study was the intralaboratory validation of four *in vitro* assays to detect skin sensitizer and to compare their predictivity using a panel of 23 test substances, including the performance standards of the LLNA.

2. Materials and methods

Test substances. The test substances for this intralaboratory validation included the 22 performance standards of the local lymph node assay according the OECD guideline 429 and 2,4,6-trinitrobenzene sulfonic acid (OECD, 2010). For these compounds results from murine LLNA as well as human patch test data are available (Kimber et al., 2003; Basketter et al., 1999b). Substances with skin sensitizing potential are 1-chloro-2,4-dinitrobenzene (98%; DNCB); 2,4,6-trinitrobenzene sulfonic acid (5% (w/v); TNBS); 2-mercaptobenzothiazole (>99%); cinnamic alcohol (98%); citral cis/trans mixture (95%); cobalt(II)-chloride (97%); ethylene glycole dimethacrylate (98%); eugenol (99%); α-hexyl cinnamic aldehyde (85%); imidazolidinylurea (95%); isoeugenol cis/trans mixture (98%); Kathon CG (1.2% 5-chloro-2-methyl-4-isothiazolin-3-one and 0.3% 2-methyl-4-isothiazolin-3-one); methylmethacrylate (99%); methyle salicylate (>99%); nickel(II)-chloride (98%); paraphenylendiamine (>99%); phenylbenzoate (99%). Test Substances without sensitizing potential are chlorobenzene (99%); isopropanol (>99%); D/L-lactic acid (90%); salicylic acid (>99%); sodium dodecylsulfate (98%); xylene (98.5%). All chemicals were purchased from Sigma–Aldrich Chemie GmbH, Germany.

3. Dendritic cell activation assay – U-937

The dendritic cell activation assay using U-937 cells, also known as Myeolid U-937 Skin Sensitization Test (MUSST), was performed as described by Python and co-workers (Python et al., 2007) with minor modifications (Fig. 1a). Briefly, U-937 cells, human monocytic leukemia cell line, were obtained from the German Resource Center for Biological Material (DMSZ; ACC 5). Cells were cultured in complete RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Germany) and 1% penicillin (100 U/mL)- streptomycin (100 μg/mL) (Biochrom AG, Germany) in T75 culture flasks (TPP, Switzerland). The cells were kept in a humidified atmosphere at 37 °C and 5% CO_2. They were subcultured every 3–4 days with an initial cell density of 1×10^5 c/mL. Cells were passaged for 5 weeks to a maximum of 15 weeks. Pre-cultures were performed by diluting the cells 1:2 for experiments at the following day and 1:4 for experiments after 2 days. For substance incubation cells were seeded in 96 well microtiter plates by pipetting 100 μL of 0.5×10^6 c/mL cell suspension. Substances were dissolved in medium (2×) or DMSO (400×). DMSO solved substances were further diluted in medium to obtain 2× stock solution. Final DMSO concentration on the cells did no exceed 0.25%. For each substance five concentrations were tested in duplicates. Concentrations were chosen according to preliminary propidium iodide (PI) cytotoxicity assays. Therefore, cells were exposed to 12 concentrations of 1:2 serially dilution starting at 2000 μg/mL for solid test compound or 1000 μg/mL for liquid test compound, respectively. Assessment of viable cells was performed according to the main experiment procedures but cells were stained using propidium iodide only. The highest tested concentration in the main experiment was 2× concentrations causing a cytotoxicity of 25% (CV75). The additional concentrations were obtained by a 1:2 dilution serie of the 2× CV75. Each concentration was run in duplicates and each experiment was performed minimum in two independent times. Cells were exposed to the test substance for 48 h under general cell culture conditions. After treatment, cells were pelleted by centrifugation at 400 g for 5 min at RT and washed once with PBS containing 5% FBS. Cells were resuspended in 100 μL PBS w/ 5% FBS and labeled for 30 min at 4 °C in the darkness with 5 μL IgG-FITC (Cat. No. 555748, BD Pharmingen, Germany) or 5 μL anti-CD86-FITC antibody (Cat. No. 555657, BD Pharmingen, Germany). Follow incubation, cells were washed twice with PBS w/ 5% FBS and resuspended in PBS. Cells nuclei were stained with propidium iodide (PI, 50 μg/mL in PBS) for 5 min at RT in the darkness. Cells were analyzed by flow cytometry using a BeckmanCoulter FC500MPL equiped with MXP software. The analysis was performed in 10,000 living cells.

4. Dendritic cell activation assay – THP-1

The dendritic cell activation assay using THP-1 cells, also known as human Cell Line Activation Test (h-CLAT), was performed as described by Ashikaga and co-workers (Ashikaga et al., 2006) and Sakaguchi and co-workers (Sakaguchi et al., 2006) with minor modifications (Fig. 1a). Briefly, THP-1 cells, human monocytic leukemia cell line, were obtained from the German Resource Center for Biological Material (DMSZ; ACC 16). THP-1 cells were cultured in complete RPMI 1640 with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany), 1% penicillin (100 U/mL)- streptomycin (100 μg/mL) (Biochrom AG, Germany) and 0.05 mM 2-mercaptoethanol (Invitrogen, Germany) in T150 culture flasks (TPP, Switzerland). The cells were kept in a humidified atmosphere at 37 °C and 5% CO_2. They were subcultured every 3–4 days with an initial cell density of 2×10^5 c/mL. Cells were passaged for 8 weeks to a maximum of 24 passages. For experiments, cells were seeded in 24 well plates (TPP, Switzerland) by adding 1×10^6 cells in 500 μL per well. Substances were dissolved in medium (2×) or DMSO (500×). DMSO solved substances were further diluted in medium to obtain 2× stock solution. Final DMSO concentration on the cells did no exceed 0.2%. For each substance eight concentrations were tested in duplicates. Concentrations were chosen according to preliminary propidium iodide (PI) cytotoxicity assays. Therefore, cells were exposed to nine concentrations of 1:2 serially dilution starting at 2000 μg/mL for solid test compound or 1000 μg/mL for liquid test compound, respectively. Assessment of viable cells was performed according to the main experiment procedures but cells were stained using propidium iodide only. The highest tested concentration in the main experiment was 1.2× CV75. The additional concentrations were obtained by a 1:1.2 dilution serie of the 1.2× CV75. Each concentration was run in duplicates and each experiment was performed minimum in two independent times. Treatment was performed by applying 500 μL of the test substance dilution to each well for 24 h. For analysis cells were transferred into 1.5 mL tubes (Eppendorf, Germany). Cells were spun down by centrifugation at 200 × g and 4 °C for 5 min and washed twice by centrifugation at 200 × g and 4 °C for 5 min with 1 mL staining buffer, PBS w/ Ca^{2+}·Mg^{2+} (Biochrom AG, Germany) and 0.1% BSA (Sigma, Germany). Cells were resuspended in 600 μL staining buffer containing 0.01% Cohn fraction (Sigma, Germany) and incubated for 15 min at 4 °C in the dark. Then cells

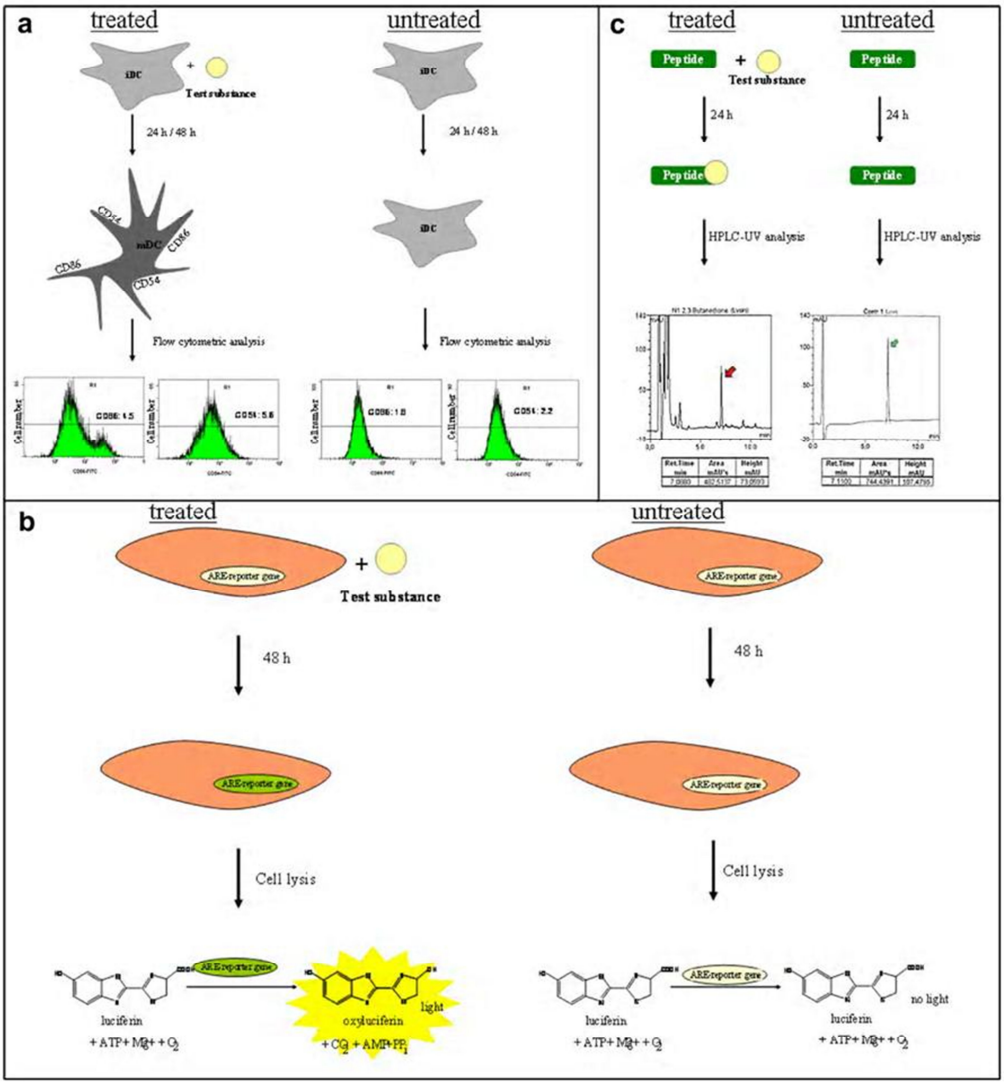

Fig. 1. Schematic representation of the *in vitro* models used in our test battery. (a) Dendritic cell activation assays (MUSST/h-CLAT): immature cells (iDC) are exposed to test substance for 48 h. Sensitizers activate iDC and lead to the maturation of DC (mDC). Consequently, specific markers are overexpressed on the cell surface (CD86 for MUSST and CD86/CD54 for h-CLAT). Cell surface markers are analyzed by flow cytometry. (c) Reporter gene analysis with KeratinoSens®: cells expressing luciferase are exposed to test substance for 48 h and analyzed according to the luciferase activity after cell lysis using SteadyGlo™ (Promega). (d) Peptide Binding Assay: cysteine and lysine peptides are exposed to test substance for 24 h and analyzed according to the peptide peak depletion using HPLC-UV technique.

were distributed in V-shaped 96 well microtiter plate (TPP, Switzerland). Each concentration replicate was split into 3 wells by pipetting 180 µL cell suspension per well. Cells were pelleted by centrifugation at 200 × g and 4 °C for 5 min and resuspended in 50 µL antibody working solution. Cells were stained for IgG1-FITC (1:16 diluted in PBS; DAKO/DAK X092701) and anti-CD54-FITC (1:16 diluted in PBS; DAKO /DAKO F7143) anti-CD86-FITC (1:7 diluted in PBS; Cat. No. 555657, BD Pharmingen, Germany). Incubation was performed at 4 °C in the dark for 30 min. Then, cells were washed twice with 200 µL staining buffer by centrifugation at 200 × g and 4 °C for 5 min and resuspended in 200 µL staining buffer. PI (50 µg/mL in PBS) was added 5 min before flow cytometric analysis and incubated at RT in the dark. Flow cytometric analysis was performed with a BeckmanCoulter FC500MPL and the MXP software.

5. KeratinoSens® assay

KeratinoSens® cell line was kindly provided by Andreas Natsch, Givaudan. This keratinocyte cell line derived from HaCaT cells carries a reporter gene for luciferase under the control of an antioxidant-response-element (ARE) and hence monitors Nrf2 transcription factor activity (Fig. 1b). The KeratinoSens® assay was performed according to Givaudan's protocol (Emter et al., 2010). Briefly, KeratinoSens® cells were cultured in complete DMEM culture medium with GlutaMAX™ -1 (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany) and 0.55 mg/mL Geneticin (G-418; Invitrogen, Germany) in T75 culture flasks (TPP, Switzerland). Cells were kept in a humidified atmosphere at 37 °C and 5% CO_2. They were subcultured every 3–4 days without exceeding a confluence greater 90%. Cells were passaged

for a maximum of 12 weeks. For chemical exposure KeratinoSens® cells were seeded in three white 96 well microtiter plates (PerkinElmer, Germany) and one transparent 96 well microtiter plate (TPP, Switzerland). Per well 1×10^4 cells in 120 μL medium containing 10% FBS w/o G-418 were added and allowed to adhere for 24 h under standard cell culture conditions. Prior to treatment, medium was aspirated and renewed by adding 150 μL medium containing 1% FBS w/o G-418. Substances were diluted in distilled water or in DMSO (final concentration of 200 mM). Stock solutions were prepared by diluting the substances in a common ratio of 1:2 with the appropriate vehicle. Substances were further diluted in a 25-fold manner by adding 10 μL stock solution to 240 μL medium containing 1% FBS w/o G-418 resulting in 4× stock solution. Treatment was performed by adding 50 μL of each 4× stock solution. For chemicals dissolved in water the DMSO level was adjusted to 1%. Substance incubation was performed under standard cell culture conditions for 48 h. Analysis were performed using Steady-Glo™ (Promega, Germany) according to manufacturer instructions and the PerkinElmer Victor^3 photometer (PerkinElmer, Germany). Cytotoxicity was assessed using MTT cytotoxicity assay. Briefly, 5 mg/mL stock solution was prepared in PBS, sterile filtrated and diluted in medium (1:10) containing 1% FBS. 200 μL of this working solution were added to each well of the transparent 96 well microtiter plate and incubated for further 2 h. For analysis medium was aspirated and cells were lysed by adding 100 μL of lysis solution (99.6 mL DMSO, Sigma–Aldrich, Germany; 10 g SDS, Sigma–Aldrich, Germany; and 0.4 mL glacial acetic acid, Merck, Germany). Absorbance was read at 570 nm with reference wavelength at 690 nm using the PerkinElmer Victor^3 photometer.

6. Direct Peptide Reactivity Assay

The Peptide Binding Assay, also known as DPRA (Direct Peptide Reactivity Assay), is a cell free, *in chemico* assay with the use of artificial model peptides according to Gerberick and co-workers (Gerberick et al., 2004) (Fig. 1c). In brief, test substances were dissolved in acetonitrile (Sigma–Aldrich, Germany) or if not soluble in acetonitrile/water mixture or water to obtain a concentration of 100 mM. Substances were freshly prepared every time before use. Cysteine model peptides (Synbiosci, Livermore CA, USA) were prepared in 0.667 mM stock solution in 100 mM phosphate buffer (100 mM NaH_2PO_4, 100 mM Na_2HPO_4, both Sigma–Aldrich, pH = 7.5) and lysine model peptides (Synbiosci, Livermore CA, USA) were prepared 100 mM ammonium acetate buffer (pH = 10.2). Substance stocks were diluted with peptide solutions in a ratio of 1:4:15 (v/v/v) substance:acetonitrile:cysteine peptide and 1:3 (v/v) substance:lysine peptide. Each substance was analyzed in triplicates for each peptide. Incubation was performed at room temperature in the dark for 24 h. Analysis of free peptides was performed by RP-HPLC (Agilent 1200 Series) with UV detection at 220 nm using a C18 Phenomex Luna® coloum (2.0 mm × 100 mm × 3 micron particle; Phenomex). Separation of peptides was achieved by isochratic flow with a flowrate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 min, followed by a rapid increase to 90% acetonitrile.

7. Results

Each assay described above was performed to assess 23 test substances of the LLNA performance standards list and TNBS. Substances were classified as skin sensitizer according to the following criteria: 1.2× or greater the expression of CD86 in the MUSST; 1.5× or greater induction of CD54 and/or CD86 in h-CLAT; 1.5× or greater luciferase expression in KeratinoSens®; and greater than 6.4% of the mean peptide depletion of cysteine and lysine peptide in the DPRA. Results were expressed as positive if the test substance was predicted as skin sensitizer and as negative if they were predicted as non-skin sensitizer. For all compounds human patch test data are available from the literature and were described offering significant skin sensitizing potential based on available clinical studies (Basketter et al., 1999b; ICCVAM, 2009). Compared to human data all cell based assays were able to predict 20 of 23 substances correctly; the in *chemico* DPRA was able to predict 21 of 23 substances correctly. The MUSST assay predicted three sensitizing compounds false negative, isoeugenol, cinnamic alcohol and α-hexyl cinnamic aldehyde (Table 1). Evaluation of data with Cooper statistics (Cooper et al., 1979) (Figs. 2 and 3) revealed that the MUSST assay offered a sensitivity of 81%, a specificity of 100%, a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 70%. The overall accuracy for MUSST assay was 87% (Fig. 2a). The h-CLAT assay predicted two sensitizing compounds false negative: imidazolinidyl urea and nickel chloride, and salicylic acid false positive. Cooper statistics revealed a sensitivity of 81%, a specificity of 86%, a PPV of 93% and a NPV of 67%. The overall accuracy for h-CLAT assay was 83% (Fig. 2b). The KeratinoSens® assay predicted three of the sensitizing compound false negative: methyl methacrylate, nickel chloride and phenyl benzoate. Cooper statistics estimated a sensitivity of 81%, a specificity of 100%, a PPV of 100% and a NPV of 70%. The overall accuracy of the KeratinoSens® assay was 87% (Fig. 2c). The *in chemico* approach DPRA predicted α-hexyl cinnamic aldehyde false negative and sodium dodecyl sulfate false positive. Cooper statistics estimated a sensitivity of 94%, a specificity of 86%, a PPV of 94% and a NPV of 86%. The overall accuracy of the DPRA was 91% (Fig. 2d).

The murine LLNA assay offered for these 23 tested compounds a sensitivity of 94%, a specificity of 86%, a PPV of 94% and a NPV 86%. The overall accuracy of the LLNA is 91%. The murine LLNA (Basketter et al., 1999b; Kimber et al., 2003) predicted nickel chloride as false negative and sodium dodecyl sulfate false positive if compared to human data. Comparison of our results with LLNA data from literature indicated that the h-CLAT assay, KeratinoSens® assay and DPRA offers the same predictivity as compared to human data (Figs. 2 and 3). The MUSST assay showed a reduced predictivity if results were compared to LLNA data than to human data. The MUSST assay predicted four of the sensitizing compounds false negative: isoeugenol, cinnamic alcohol, α-hexyl cinnamic aldehyde and sodium dodecyl sulfate, and nickel sulfate false positive. Cooper statistics displayed that the MUSST assay offered a sensitivity of 75%, a specificity of 86%, a PPV of 92% and a NPV of 60%. The overall accuracy for MUSST assay was about 78% (Fig. 3a).

8. Discussion

In this study we report the intra-laboratory validation of four *in vitro* assays: the *in chemico* Peptide Binding Assay (DPRA; (Gerberick et al., 2004)), the reporter gene based assay – KeratinoSens® assay from Givaudan with an ARE-dependent luciferase reporter gene for Nrf2 activity and the dendritic cell activation assays using the cell lines U-937 cells (MUSST assay) and THP-1 cells (h-CLAT assay). The performance standards of the LLNA (OECD, 2010) were used as test substances comprising 16 sensitizing and 7 non-sensitizing compounds.

The results of each assay were compared to literature data of human patch tests and murine LLNA and were evaluated with so called Cooper statistics. Results for human data where taken from literature (Basketter et al., 1999b). Basketter and co-workers referred to other publications (Gerberick et al., 2000; Kimber et al., 1994; Kligman, 1966; Basketter et al., 1996; 1994; 1999a; De Groot et al., 1994; Rietschel and Fowler, 1995) where those data were based on available clinical studies only and offered significant

Table 1
Summary of the data obtained from the *in vitro* test systems, the human patch test and murine LLNA data were obtained from the literature. Substances indicated as (+) were rated positive and those indicated as (−) were rated as negative.

Substance	Literature *In vivo*		Experimental data *In vitro*			
	Human	LLNA	U-937	THP-1	KeratinoSens	DPRA
1-Chloro-2,4-dinitrobenzene	+	+	+	+	+	+
2-Mercaptobenzothiazole	+	+	+	+	+	+
2,4,6-Trinitrobenzenesulfonic acid	+	+	+	+	+	+
4-Phenylenediamine	+	+	+	+	+	+
Cinnamic alcohol	+	+	−	+	+	+
Citral	+	+	+	+	+	+
Cobalt chloride	+	+	+	+	+	+
Ethylene glycol dimethacrylate	+	+	+	+	+	+
Eugenol	+	+	+	+	+	+
Hexyl-cinnamic aldehyde	+	+	−	+	+	−
Imidazolidinyl urea	+	+	+	−	+	+
Isoeugenol	+	+	−	+	+	+
Kathon CG	+	+	+	+	+	+
Methyl methacrylate	+	+	+	+	−	+
Nickel chloride	+	−	+	−	−	+
Phenyl benzoate	+	+	+	+	−	+
Chlorobenzene	−	−	−	−	−	−
Isopropanol	−	−	−	−	−	−
DL-lactic acid	−	−	−	−	−	−
Methyl salicylate	−	−	−	−	−	−
Salicylic acid	−	−	−	+	−	−
Sodium lauryl sulfate	−	+	−	−	−	+
Xylene	−	−	−	−	−	−

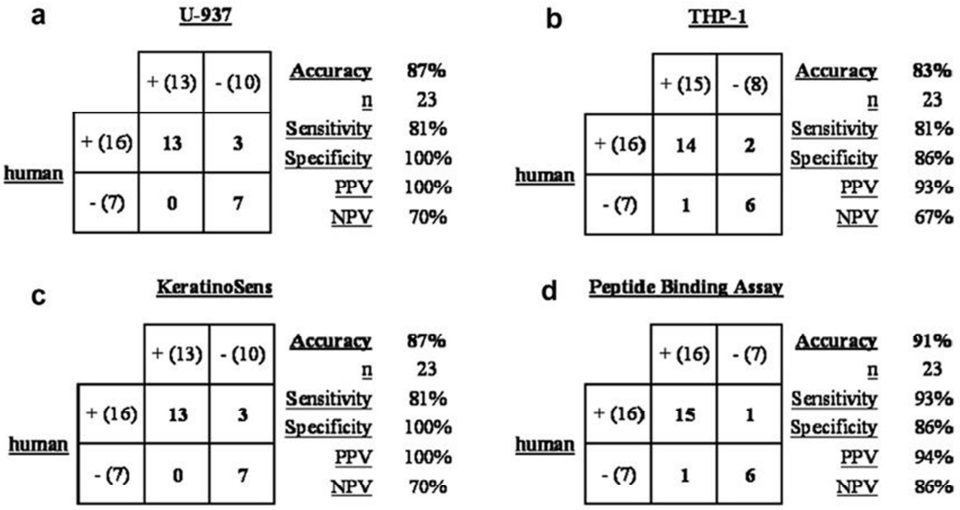

Fig. 2. Predictivity of each assay in comparison to human patch test data. Evaluation was performed using the Cooper statistics (Cooper et al., 1979). Substances predicted as sensitizers are indicated as (+) and substances predicted as non-sensitizer as (−).

evidence for skin sensitization (Basketter et al., 1999b). In summary the assays offered accuracies between 83% and 91% compared to human patch test data and between 78% and 91% compared to LLNA data. Remarkably, the DPRA showed in both comparisons the highest accuracy of 91%, followed by the KeratinoSens® assay with 87% and h-CLAT assay with 82%. The MUSST assay had an accuracy of 87% when compared to human data, but only 78% accuracy when compared to the LLNA data. It is important to notice that these predictivities were performed for 23 substances only and that they could be different if a larger (and/or different) set of substances is used to assess the predictivities. Nevertheless, it is remarkable that the parallel testing of the four different methods with the same set of 23 substances yielded predictivities around 80% for the single assays.

Our data indicate that the combination of different assays, such as dendritic cell activation (MUSST or h-CLAT assay) with DPRA and/or KeratinoSens® assay, could create a prediction model with excellent predictivity. According to the hypothesis that a single assay will not be able to replace the animal testing approaches we combined the above mentioned methods in pairs (data not shown). This led to an increased accuracy of each approach with a striking result for the combination of MUSST assay with the KeratinoSens® assay: In this simple test battery no false positive or negative prediction was done and the accuracy would be 100% for the 23 test

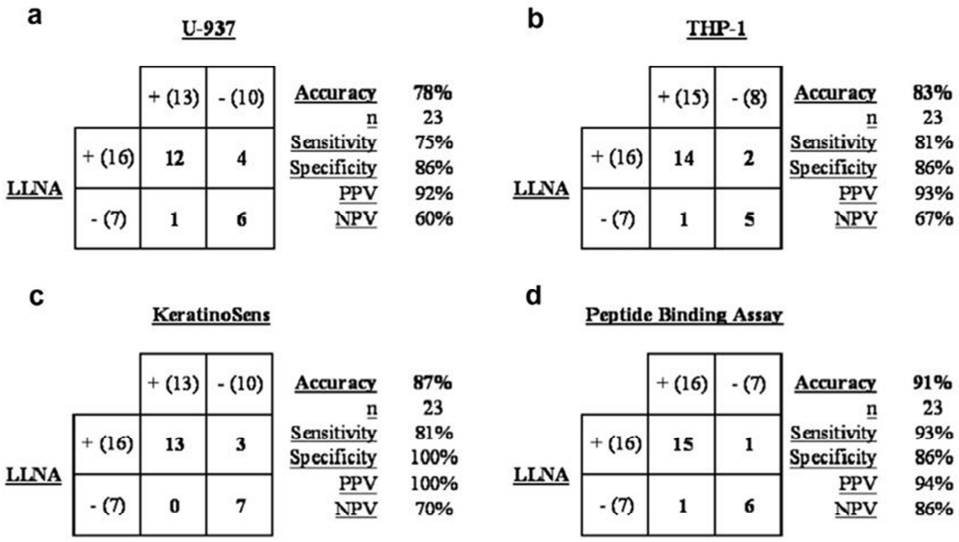

Fig. 3. Predictivity of each assay in comparison to murine local lymph node data (LLNA). Data analysis was performed using the Cooper statistics (Cooper et al., 1979). "+" indicates the number of substances predicted as sensitizer and "−" the number of substances predicted as non-sensitizer of experimental or literature data. This applies for each assay.

compounds tested. Our data for the single assays and the pairing of assays could be further validated with a lager set of test compounds.

As described above the penetration of test substance through the skin is an important factor of the skin sensitization process, since it leads to the bioavailability of haptens in the viable dermis. The stratum corneum is a barrier against the entry of noxious materials, but substances of low molecular weight (<500 Da) with lipophilic properties are able to pass this barrier (Basketter et al., 1999b). Nevertheless, the topically applied dose may not reflect the actual dose in the dermis. Currently, the arising *in vitro* approaches using cell culture do not consider the natural physical barrier that represents the stratum corneum and the reduced bioavailability of test substances *in vivo*. Therefore, there is a need for further development of test strategies that could evaluate dermal penetration on *in vitro* skin models and *in silico* processing, such as QSAR, by which the chemical structure of a compound could be quantitatively correlated to dermal penetration (Basketter et al., 1999b; Pendlington et al., 2008).

The epidermal inflammatory response induced by skin sensitizers facilitates the response of dendritic cells and T cells via the release of cytokines like IL-18. Corsini and co-workers proposed a test strategy using the keratinocyte cell line NCTC 2544 and the IL-18 release to identify skin sensitizer and to differentiate them from irritants and respiratory allergens (Corsini et al., 2009). Coquette and co-workers described the possibility to distinguish irritants and skin sensitizer by considering the IL-1α and IL-8 ratio in reconstructed skin models (Coquette et al., 2003). Including inflammatory responses in a tiered test strategy for skin sensitization could further improve the testing strategy and help to discriminate skin sensitizers from irritants.

Isoeugenol and cinnamic alcohol are prohaptens reported to be skin sensitizers without direct reactivity towards proteins (Smith and Hotchkiss, 2001; Barratt and Basketter, 1992; Basketter, 1992; Lepoittevin, 2006). Both chemicals were predicted as true positive in the h-CLAT assay, KeratinoSens and DPRA assay. Whereas the MUSST assay, using the cell line U-937, was not able to predict isoeugenol and cinnamic alcohol as skin sensitizer. If cellular systems like dendritic like cell lines do not offer any metabolic capacity they would not be sufficient to predict prohaptens (Chipinda et al., 2011). Therefore the metabolic capacity of the cellular systems test systems should be investigated. A lack of metabolic capacity can be compensated. Schreiner and co-workers for instance, established a co-culture of keratinocytes and dendritic cells able to detect pro-haptens like isoeugenol as sensitizers (Schreiner et al., 2008). Chipinda and co-workers featured the THP-1 based testing system with liver S9-mix of aroclor-induced rats (Chipinda et al., 2011). The use of skin models could include the metabolic activation of the various cell types of the skin and dermal penetration in the different layers of the skin (Oesch et al., 2007; Pendlington et al., 2008; Jaeckh et al., 2011). Surprisingly, the *in chemico* assay DPRA was able to detect all four prohaptens (2-mercaptobenzothiazole, eugenol, isoeugenol and cinnamic alcohol). This may be attributed to a non-enzymatic activation – as discussed for isoeugenol (Gerberick et al., 2009) – or may, indeed, reflect false positives in this assay.

A prediction model for skin sensitization should further be able to classify test substances not only as sensitizers but also as weak, moderate, strong or extreme sensitizer. The LLNA classifies skin sensitizers according to the test concentrations provoking a proliferation of T cells with a fold induction greater that 3× compared to vehicle control and with set thresholds for categorization as weak, moderate, strong and extreme sensitizer (Kimber et al., 2003). Jowsey and co-workers have published a hypothetical prediction model where dose–response and a relative activity in test systems are assigned with a score for a classification of skin sensitizers (Jowsey et al., 2006).

Until now there is no applicability domain for the assays presented. To define the applicability domain it would be useful to increase the substance panel to include chemicals with broader physico-chemical properties. Furthermore, in order to show the robustness and predictivity of these assays, it may be required a (pre)validation with a substance panel including blindly coded test substances. Currently, MUSST, h-CLAT and DPRA are under pre-validation by ECVAM and the KeratinoSens® will be also submitted for its pre-validation.

Our study is the first one in which the four assays were performed in parallel with a defined panel of 23 substances and the direct correlation to human patch test and murine LLNA data, making it a reliable approach to compare their potential to predict skin

sensitizers. Currently, we enhanced our substance panel up to 57 compounds to confirm our findings.

Conflict of Interest Statement

Caroline Bauch, Susanne N. Kolle, Eric Fabian, Tzutzuy Ramirez, Bennard van Ravenzwaay and Robert Landsiedel are employees of BASF SE, a chemical company intending to use the toxicological assays to test their commercial products. These authors are, however, not aware of any conflict of interest.

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6 DEFINITION OF A TEST STRATEGY

6.1 PUTTING THE PARTS TOGETHER: COMBINING IN VITRO METHODS TO TEST FOR SKIN SENSITIZING POTENTIALS

Bauch C.; Kolle S. N.; Ramirez, T.; Eltze, T.; Fabian E.; Mehling, A.; Teubner, W.; van Ravenzwaay, B; Landsiedel, R.: *Putting the parts together: Combining in vitro methods to test for skin sensitizing potentials.*; **Regulatory Toxicology and Pharmacology 63 (3)**, p.489-504; DOI: 10.1016/j.yrtph.2012.05.013; 2012

This chapter focuses on the continued validation of the four assays described in the previous chapter (Chapter 5). With the manuscript "Putting the parts together: Combining *in vitro* methods to test for skin sensitizing potential" an extended study with a total of 54 chemicals are described. In addition to the assays DPRA, KeratinoSens, MUSST and h-CLAT a second reporter gene based assay was performed, so-called LuSens assay. LuSens is similar to KeratinoSens in its principle and a probable alternative for it. The main difference between LuSens and KeratinoSens cell line are the underlying ARE elements of the used genes. LuSens carries the ARE element of the rat NADPH:quinine oxidoreductase 1 gene and a puromycin resistance gene as a selection marker. The *in silico* method OECD QSAR Toolbox (Version 2.0 2010) was used for an initial prediction of protein reactive properties and for a possible reaction mechanism. The second objective of this study was to develop a simple model to combine results of the individual assays of this testing battery in order to reflect the steps of the AOP of skin sensitization to achieve a high predictivity of skin sensitization potentials in humans.

Due to technical issues during this study few test substances had to be excluded from the initial list of 59 test substances: 1-chlorobenzene, 1-bromobutan and geraniol, since they precipitated in the cell culture media during the performance of MUSST and h-CLAT. The solubility could not be increased as by higher DMSO concentrations (>0. 5% of DMSO) activation of DC was recorded. In addition the test substances Tween 80 and Triton-X 100 seemed to interfere with the performance of the used flow cytometer. Although cells were washed to remove remaining test substance the system detected unspecific air bubbles and created error messages. The final list of 54 chemicals represents various chemical classes and with different proposed reaction mechanism. Although most chemicals have been described in the literature, reliable human patch test data were only available for 50 compounds and LLNA data only for 53, hence calculated prediction could only be performed for this number of chemicals.

The DPRA, KeratinoSens, MUSST and h-CLAT assays were carried out as described in the previous chapter and had the following number of correct predictions compared to human data as following: 43, 40, 43 and 38 out of 50 using DPRA, KeratinoSens, MUSST and h-CLAT, respectively. Compared to LLNA data the number of correct predictions was 42, 43, 39 and 39 out of 53 using DPRA, KeratinoSens, MUSST and h-CLAT,

respectively. The reporter gene cell line HaCaT NQO1 was prepared in collaboration with Dr. Christopher Wruck at RWTH Aachen and was further established as LuSens at BASF SE and was integrated in an assay with the same name. The LuSens predicted 42 out of 50 or 41 out of 53 correctly, compared to human data or LLNA data respectively.

For an initial prediction of sensitization potential the OECD QSAR Toolbox was used. The software identifies chemical structure elements likely to react with proteins and proposes a possible reaction mechanism. The OECD QSAR toolbox can include a metabolism feature to analyze possible metabolites, which could predict pro-haptenic substances, albeit this feature was not used in this work. Nevertheless, the OECD QSAR Toolbox predicted 40 out of 50 or 37 out of 53 substances correctly if compared to human or LLNA data, respectively.

Calculation of Cooper statistics if compared to human patch test data (or LLNA data) indicated sensitivities of the single assays between 64 % and 89 % (56 % and 83 %) and specificities ranged between 73 % and 100 % (71 % and 100 %). Accuracies for the single assays were between 76 % and 86 % (74 % and 81 %.).

Combination of two assays changed the predictivity for all combinations compared to human data (or LLNA data). A substance was rated as sensitizer if at least one of two assays gave a positive result. Thus sensitivities have been increased up to a range between 93 % and 100 % (86 % and 94 %,) for various combinations of two assays, and specificities decreased down to a range of 56 % to 82 % (53 % to 92 %).

The most promising combination was obtained with three assays with the application of a two out of three rule. A chemical was classified as a probable sensitizer if any two of the selected three assay gave positive results. Combination of DPRA, LuSens or KeratinoSens with MUSST resulted in sensitivities of 93 % (81 %), specificities of 95 % (88 %) and accuracies of 94 % (83%) if compared to human data (or LLNA data) regardless whether combination with LuSens or KeratinoSens was applied.

To further describe a testing strategy a selection of assays of this testing battery was performed to represent three consecutive early steps of the AOP of skin sensitization. DPRA and LuSens (or KeratinoSens) represent haptenization and KC activation. Combination of the results of both assay (potential sensitizer if one assay gives a positive result) allows for predictions of non sensitizers with a sensitivity of 100%. This implies that a skin sensitization potential can be excluded if there is neither haptenization nor KC and that activation for the combination of these two steps appears to be a necessary step of skin sensitization. Likewise, the MUSST represents DC activation and resulted in predictions of sensitizers with a specificity of 100%. This implies that in many cases DC activation is sufficient to predict skin sensitizing potential.

In few cases the results of these three assays can be inconclusive (e.g. DPRA or LuSens are positive, but MUSST is negative) In such cases a weight of evidence (WoE) is proposed: If any two of these three assays are positive the substance is rated as skin sensitizer. The h-CLAT can replace the MUSST equally, although its

specificity was compared to MUSST assay only 77%. The figure of the manuscript was updated and is shown in Figure 4.

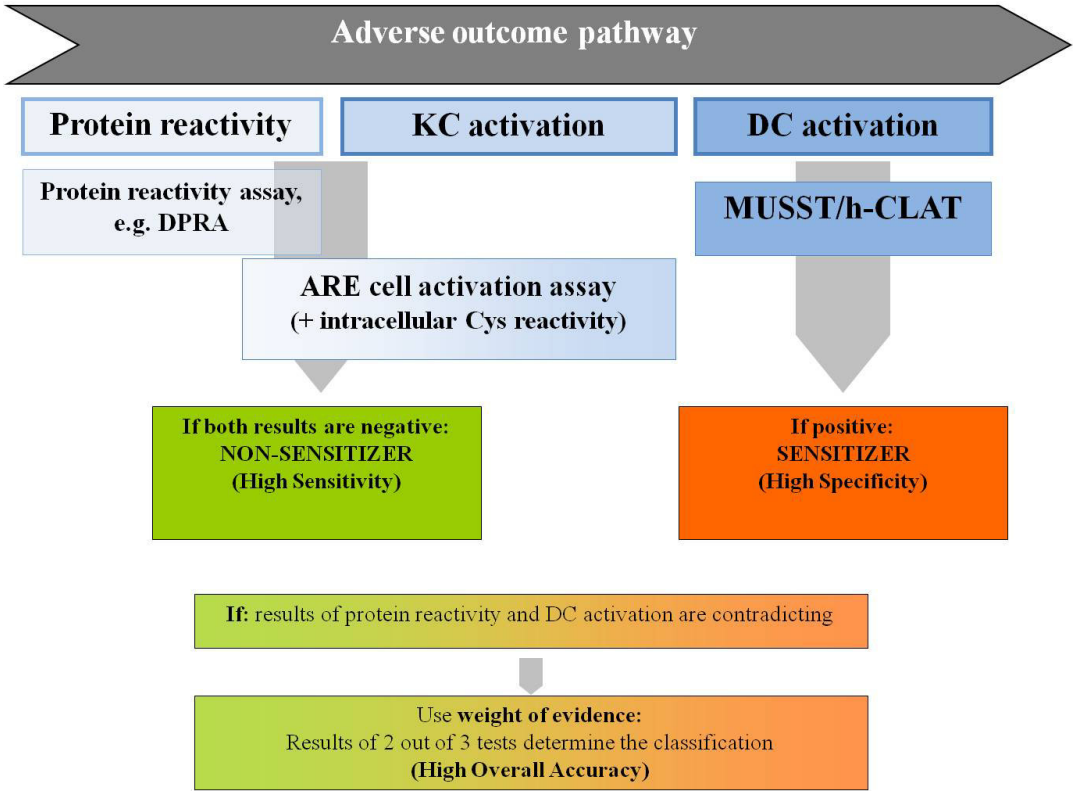

Figure 4. Updated figure of proposed testing strategy (Bauch et al. 2012)
According to the AOP for skin sensitization was a test strategy postulated using DPRA to predict protein reactivity, Nrf2-ARE-reporter gene analysis (either LuSens or KeratinoSens) for KC activation and indirect protein reactivity for a high probable exclusion of sensitizing potential and the cell activation assays MUSST or h-CLAT to predict DC activation and high probable prediction of sensitizing potential. In case of contradicting results a Weight of Evidence is proposed offering a high overall accuracy.

The validation of KeratinoSens as well as the establishment and validation of the LuSens cell line were part of my PhD project with assay performance and data evaluations and were solely performed by myself. I contributed the performance of MUSST and h-CLAT and performed data evaluation. Literature research according reaction mechanism and the arrangement of results and writing of the manuscript were also my duty. The data used from the DPRA assay were already existing and data evaluation for this work had to be done.

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Putting the parts together: Combining *in vitro* methods to test for skin sensitizing potentials

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ABSTRACT

Allergic contact dermatitis is a common skin disease and is elicited by repeated skin contact with an allergen. In the regulatory context, currently only data from animal experiments are acceptable to assess the skin sensitizing potential of substances. Animal welfare and EU Cosmetic Directive/Regulation call for the implementation of animal-free alternatives for safety assessments. The mechanisms that trigger skin sensitization are complex and various steps are involved. Therefore, a single *in vitro* method may not be able to accurately assess this endpoint. Non-animal methods are being developed and validated and can be used for testing strategies that ensure a reliable prediction of skin sensitization potentials. In this study, the predictivities of four *in vitro* assays, one *in chemico* and one *in silico* method addressing three different steps in the development of skin sensitization were assessed using 54 test substances of known sensitizing potential. The predictivity of single tests and combinations of these assays were compared. These data were used to develop an *in vitro* testing scheme and prediction model for the detection of skin sensitizers based on protein reactivity, activation of the Keap-1/Nrf2 signaling pathway and dendritic cell activation.

1. Introduction

As the interface between the environment and the body, the skin is continuously exposed to environmental insults, pathogens and xenobiotics. In particular, consumers and workers are often exposed to chemicals via cosmetic and household products or in industrial settings on a daily basis and to a significant degree. One of the adverse effects that can occur as a result of skin exposure to xenobiotics is contact sensitization, the clinical manifestation of which is allergic contact dermatitis (ACD). The principle objective of toxicological testing is to provide a basis for the assessment of hazards and to identify potential risks from use and handling of products, such as chemicals or cosmetic formulations, thus ensuring that adverse effects to human health do not occur. The evaluation of the sensitization potential of a substance has therefore been of central importance for hazard and risk assessments for decades. Currently, most toxicological endpoints in the regulatory context are assessed via animal testing. This is also the case for the sensitization potentials for which generally only the animal studies described in OECD 406 (guinea pig tests according to Buehler or Magnusson & Kligman) or OECD 429 and OECD 442 (murine local lymph node assays, LLNA) are accepted by the regulatory bodies.

The increasing emphasis on the ethics of animal testing has manifested itself in a regulatory context in the recent chemicals legislation on the registration, evaluation, authorization and restriction of chemicals (REACH (EU, 2006)) and even more so in

Abbrevations: ARE, antioxidant response element; AUC, area under the curve; C.C, control cells; CV75, concentration reducing viability to 75%; DC, dendritic cells; DMSO, dimethyl sulfoxide; DNCB, 1-chloro-2,4-dinitrobenzene; DPRA, Direct Peptide Reactivity Assay; ECHA, European Chemicals Agency; ECVAM, European Centre for the Validation of Alternative Methods; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; h-CLAT, human Cell Line Activation Test; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Keap-1, Kelch-like ECH-associated protein 1; ITS, integrated testing strategy; LLNA, local lymph node assay; MCI/MI, mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; mMUSST, modified myeloid U937 dendritic cell activation-based skin sensitization test); NADPH, nicotinamide adenine dinucleotide phosphate; Nrf2, nuclear factor (erythroid-derived 2)-like factor 2; OECD, Organisation for Economic Cooperation and Development; PBS, phosphate buffered saline; PI, propidium iodide; QSAR, quantitative structure-activity relationship; REACH, Registration Evaluation Authorisation and Restriction of Chemicals; RLU, relative luminescent unit; RP HPLC, reverse phase high performance liquid chromatography; RT, room temperature; SDS, sodium dodecyl sulfate; T.C, treated cells; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

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the amendments of the European Cosmetics Directive (Council Directive 76/768/EEC, 1976; EU, 2003). With the advent of REACH, an enormous number of animal tests were estimated to be necessary to address the various toxicological endpoints (Hartung and Rovida, 2009). Hazard assessment of the sensitization potential is one of the endpoints in the base set needed for all chemicals registered under REACH. REACH mandates that every effort must be made to avoid animal testing and that animal testing should only be conducted as a last resort. Usage of validated and adequately documented *in vitro* methods for the adaptation of the standard testing regime is authorized in Annex XI of the REACH legislation. Recently, the European Chemicals Agency (ECHA) published a guideline on the use of alternative to animal testing under REACH to address this issue (European Chemicals Agency, 2011). The 7th Amendment of the Cosmetics Directive (Council Directive 76/768/EEC, 1976; EU, 2003) stipulates a progressive phasing out of animal tests for the purpose of safety assessments of cosmetics and includes a concomitant marketing ban. An animal testing ban on finished cosmetic products has already been in place since 2004. A testing ban on chemicals for the purposes of the Cosmetics Directive for acute toxicity testing has been in place since 2009 with the final deadline for animal testing for the more complex toxicological endpoints including skin sensitization foreseen for 2013. A full marketing ban for cosmetics containing ingredients tested for the purpose of the Cosmetics Directive/Regulation regardless of whether non-animal alternative methods exist is foreseen for 2013. For the safety of consumers and workers, it is therefore important to develop prediction models that do not involve animal testing and yet still reliably assess toxicological endpoints.

The ethical and legislative demands have triggered intense research in the field of alternative methods (e.g. reviewed in Adler et al., 2011; Goebel et al., 2012; Mehling et al., in press). With the current innovation in this field, a number of scientific advancements have been made to assess skin sensitization. Various promising methods have been developed and four methods are currently in the process of undergoing formal validation at the European Centre for Validation of Alternative Methods (ECVAM). These test methods have already been extensively studied and address a number of key steps in the skin sensitization process: Protein-binding/haptenization (e.g. the Direct Peptide Reactivity Assay, DPRA) (Gerberick et al., 2004), induction of the Kelch-like ECH-associated protein 1 (Keap-1)/nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) pathways in keratinocytes (e.g. KeratinoSens™ assay) (Emter et al., 2010), and the activation of antigen presenting cells such as dendritic cell-like cell lines (modified myeloid U937 dendritic cell activation test, (mMUSST) or the human Cell Line Activation Test, h-CLAT) (Ashikaga et al., 2006; Bauch et al., 2011; Nukada et al., 2011; Sakaguchi et al., 2006, 2007). However, due to the complexity of the sensitization process, it is unlikely that a single assay will be sufficient to adequately assess the sensitization potential (Corsini et al., 2009; dos Santos et al., 2009). Therefore, results from test batteries will have to be incorporated into integrated testing strategies (ITS) in order to obtain the best possible predictivity for skin sensitization without the use of animals (Jaworska et al., 2011).

A number of substances, e.g. eight surfactants: (Ball et al., 2011) and 23 substances (Bauch et al., 2011) have been tested in parallel in the above mentioned assays. The purpose of the present study was to expand the data base of comparative testing available to the scientific community and to describe the path to a prediction model for the assessment of the skin sensitizing potential which could be used in a regulatory setting. For this purpose, the DPRA, KeratinoSens™, h-CLAT and mMUSST assays, which address three different key events of the skin sensitization process, namely peptide binding, activation of Keap-1/Nrf2 signaling pathway and dendritic cell activation, were performed with 54 test substances. Taking into account that certain substances require metabolic activation to acquire their sensitizing potential, a selection of pro- and prehaptens of different chemical classes were included. An additional *in-house* model which gives readout on the Keap-1/Nrf2 pathway, the LuSens assay, was also evaluated. In addition, the OECD quantitative structure–activity relationship (QSAR) Toolbox Version 2.0 was used to assess protein reactivity and to gain insights into the possible mechanisms involved. The predictivity of individual assays and combinations thereof were compared with the aim of identifying a prediction model which would allow a partial or complete replacement of animal testing for sensitization testing for a wide range of substances.

2. Materials and methods

2.1. Test substances

Originally 59 test substances were selected for this study of which 5 (*Chlorobenzene, 1-bromobutane, geraniol, Tween 80 and Triton-X 100)* were excluded from further evaluation and testing due to technical reasons (see discussion). Table 1 summarizes the test substances and their properties. It includes molecular weights, purities, supplier, CAS number, chemical classes, proposed reaction mechanisms, information about known pro- or prehapten properties, human literature data, EC3 (%) value of LLNA data and the respective literature reference. The mMUSST, h-CLAT, KeratinoSens™ and DPRA data for 23 substances has been previously published (Bauch et al., 2011) and was included for further assessment of the test battery. Of the 54 substances, 34 are considered to be skin sensitizers and 19 nonsensitizers according to LLNA data (no LLNA data was available for one substance); 21 of the substances used are performance standards recommended to assess the reliability of modifications made to the local lymph node assay test protocol (ICCVAM, 2009; OECD, 2010).

2.2. LLNA data and human data

For all 54 substances, results from animal tests, primarily from the murine LLNA, and/or human data were available and described in the literature (Andersen and Frankild, 1997; Aptula and Roberts, 2006; Basketter et al., 2008, 1999b; Casati et al., 2009; Gerberick et al., 2001, 2005; Hansson and Thoerneby-Andersson, 2003; Kimber et al., 2003; Kligman, 1966; Kreiling et al., 2008; Natsch et al., 2008; OECD, 2010; Patlewicz et al., 2008; Robinson, 1989; Ryan et al., 2000; Schneider and Akkan, 2004; SSCP/0863/05 2005; Uter et al., 2007). No LLNA data were available for hexadecyltrimethylammonium bromide and glucose, whereas no suitable human patch test data were available for 2,4,6-trinitrobenzensulfonic acid, 2,3-butandione and glucose. For glucose it was assumed to be negative in humans and mice as it is part of cellular metabolism and exposure is high. Where available, human data were used to compare with the LLNA and assessment of sensitization potential was based solely on the data reported for the available clinical studies and the assessments made by other authors (Basketter et al., 1999a,b; De Groot et al., 1994; Rietschel and Fowler, 1995). Results and the literature references are listed in Table 1.

2.3. OECD QSAR Toolbox Version 2.0

The protein binding module contained in the OECD QSAR Toolbox (Version 2.0, 2010, http://www.oecd.org/document/54/0,3746,en_2649_34379_42923638_1_1_1_1,00.html) was used to make an initial *in silico* assessment of the sensitization potential of the test substances, to identify whether they contained any

Table 1
Overview of test substances.

Substance	molecular weight	purity	supplier	CAS #	chemical class	mechanism	Literatur	Pro/Prehapten	LLNA Performance Standard?	Human	Literature	EC3 (%)	potency	LLNA	Literature
oxazolone	217.22	98 %	Sigma	15646-46-5	oxazole	Acyl-transfer	Patlewicz et al. 2008		No	+	Basketter et al. 1999	0.003	extreme	+	Kimber et al. 2003
MCI/MI	60.1	99 %	Sigma-Aldrich	26742-55-4 / 2682-20-4	aryl halide	Michael acceptor	Patlewicz et al. 2008		Yes	+	Basketter et al. 1999	0.009	extreme	+	Kimber et al. 2003
p-benzoquinone	108.1	99 %	Sigma-Aldrich	106-51-4	quinone	Michael acceptor	Patlewicz et al. 2008		No	+	Basketter et al. 1999	0.0099	extreme	+	Gerberick et al. 2005
1-chloro-2,4-dinitrobenzene	202.6	95 %	Aldrich	97-00-7	nitroaromatic / aryl halide	SNAr	Patlewicz et al. 2008		Yes	+	Basketter et al. 1999	0.049	extreme	+	Kimber et al. 2003
4-phenylenediamine	108.14	97 %	Sigma	106-50-3	arylamine	Pro-Michael acceptor	Patlewicz et al. 2008	pre	Yes	+	Basketter et al. 1999	0.16	strong	+	Gerberick et al. 2005
propyl gallate	212.2	99 %	Fluka	121-79-9	benzoate (ester) / phenolic	Acyl-transfer	author's data		No	+	Basketter et al. 1999	0.32	strong	+	Natsch and Emter 2008
2,4,6-trinitrobenzenesulfonic acid	293.17	98 %	Sigma	2508-19-2	nitroaromatic	SNAr	author's data		No	n.d.	no suitable data available	0.36	strong	+	Robinson et al. 1989
phthalic anhydride	148.1	5 %	Fluka	85-44-9	aromatic carboxylic acid anhydride	Acyl-transfer	Aptula and Roberts, 2006		No	+	Basketter et al. 2001	0.36	strong	+	Kimber et al. 2003
formaldehyde >36% (1% in DMSO)	220.35	99%	Sigma	50-00-0	aliphatic aldehyde	Schiff base	Patlewicz et al. 2008		No	+	Basketter et al. 1999	0.61	strong	+	Gerberick et al. 2005
methyldibromo glutaronitrile	256.94	98 %	Aldrich	35691-65-7	nitrile	Pro-Michael acceptor	Canati et al. 2009		No	+	SCCP 2005	0.9	strong	+	Basketter et al. 2008
isoeugenol	388.59	99 %	Sigma-Aldrich	97-54-1	phenylpropanoid	Pro-Michael acceptor	Aptula and Roberts, 2006	pro/pre	Yes	+	Basketter et al. 1999	1.5	moderate	+	Kimber et al. 2003
diethyl maleate	129.84	99 %	Sigma	141-05-9	carboxylic acid ester	Michael acceptor	Patlewicz et al. 2008		No	+	Ryan et al. 2000	2.1	moderate	+	Kimber et al. 2003
ethylene diamine	90.08	99 %	Sigma	107-15-3	alkyl amine / alkylenediamine	pro-Schiff base	Patlewicz et al. 2008	pro	No	+	Basketter et al. 1999	2.2	moderate	+	Gerberick et al. 2005
benzylidene acetone	93.13	99 %	Aldrich	122-57-6	keton / aromat	Michael acceptor	Aptula and Roberts, 2006		No	+	Schneider and Aldian 2004	3.7	moderate	+	Gerberick et al. 2005
cobalt chloride	152.2	98 %	Aldrich	7791-13-1	heavy metal salt	coordination bonds	author's data		Yes	+	Basketter et al. 1999	4.8	moderate	+	OECD 2010
2-phenylpropionaldehyde	134.18	99 %	Sigma-Aldrich	93-53-8	aldehyde	Schiff base	author's data		No	+	Schneider and Aldian 2004	5.911	moderate	+	Natsch and Emter 2008
α-hexyl-cinnamic aldehyde	216.3	98 %	Aldrich	101-86-0	aldehyde	Michael acceptor	Patlewicz et al. 2008		Yes	+	Basketter et al. 1999	8	moderate	+	Kimber et al. 2003
tartaric acid	150.09	98 %	Aldrich	133-37-9	aliphatic carboxylic acid / glycol	non-binding	Patlewicz et al. 2008		No	-	Basketter et al. 1999	8.7	moderate	+	Gerberick et al. 2005
2-mercaptobenzothiazole	167.24	99.80%	Sigma	149-30-4	thiazole / heterocyclic	Acyl-transfer	Patlewicz et al. 2008		Yes	+	Basketter et al. 1999	9.7	moderate	+	Kimber et al. 2003
2,3-butanedione	86.09	10.15 %	Sigma	431-03-8	epoxide	Schiff base	Patlewicz et al. 2008		No	n.d.	no suitable data available	11	weak	+	Gerberick et al. 2005
citral	134.18	98.10%	BASF	5392-40-5	aldehyde / terpenoids	Schiff base	Patlewicz et al. 2008		Yes	+	Basketter et al. 1999	13	weak	+	Kimber et al. 2003
eugenol	198.2	90 %	Fluka	97-53-0	phenylpropanoid	Pro-Michael acceptor	Patlewicz et al. 2008	pro	Yes	+	Basketter et al. 1999	13	weak	+	Gerberick et al. 2005
farnesal	164.2	>96.5%	Sigma	19317-11-4	sesquiterpenalkohol	Schiff base	Patlewicz et al. 2008		No	n.d.	no suitable literature	13	weak	+	Gerberick et al. 2005
sodium lauryl sulfate	288.38	99 %	Sigma-Aldrich	151-21-3	alkyl sulfate	non-binding	Patlewicz et al. 2008		Yes	-	Basketter et al. 1999	14	weak	+	Gerberick et al. 2005
4-allylanisole	148.2	95 %	Aldrich	140-67-0	aryl alkyl ether / phenylpropanoid	Pro-Michael acceptor	Patlewicz et al. 2008	pro	No	n.d.	no suitable data available	18	weak	+	Gerberick et al. 2005
hydroxycitronellal	164.45	99%	Sigma	107-75-5	terpene aldehyde	Schiff base	Patlewicz et al. 2008		No	+	Basketter et al. 1999	20	weak	+	Kimber et al. 2003
phenyl benzoate	198.22	99.4 %	Fluka	93-99-2	benzoate (ester)	Acyl-transfer	Patlewicz et al. 2008		Yes	+	OECD 2010	20	weak	+	Gerberick et al. 2005

(*continued on next page*)

Table 1 (*continued*)

Substance	molecular weight	purity	supplier	CAS #	chemical class	mechanism	Literatur	Pro/Prehapten	LLNA Performance Standard?	Human	Literature	EC3 (%)	potency	LLNA	Literature
cinnamic alcohol	112.6	97 %	Alfa Aesar	104-54-1	aromat	Pro-Michael acceptor	Patlewicz et al. 2008	pro	Yes	+	OECD 2010	21	weak	+	Gerberick et al. 2005
imidazolidinyl urea	172.27	99%	Sigma	39236-46-9	imidazoline	Acyl-transfer	Patlewicz et al. 2008		Yes	+	Basketter et al. 1999	24	weak	+	Gerberick et al. 2005
undecylenic acid	148.28	>95%	Fluka	112-38-9	carboxylic acid	non-binding	author's data		No	+	Kreiling et al. 2008	25	weak	+	Kreiling et al. 2008
geraniol	116.07	>85%	SAFC	106-24-1	terpene alcohol	pro-Schiff base	Patlewicz et al. 2008	pro	No	+	ICCVAM 1999	26	weak	+	Gerberick et al. 2005
ethylene glycol dimethacrylate	60.1	99 %	Aldrich	97-90-5	acrylate	Michael acceptor	Patlewicz et al. 2008		Yes	+	Basketter et al. 1999	35	weak	+	Kimber et al. 2003
pyridin	92.1	99 %	Sigma	110-86-1	aromatic heterocyclic	non-binding	Patlewicz et al. 2008		No	-	Basketter et al. 1999	71.2	weak	+	Gerberick et al. 2005
aniline	160.172	99 %	Sigma	62-53-3	arylamine	SNAr	Patlewicz et al. 2008	pro	No	+	Basketter et al. 1999	89	weak	+	Gerberick et al. 2005
methyl methacrylate	100.12	99 %	SAFC	80-62-6	acrylate	Michael acceptor	author's data		Yes	+	OECD 2010	90	weak	+	OECD 2010
xylene	106.16	95 %	Aldrich	1330-20-7	aromatic hydrocarbon	non-binding	author's data		Yes	-	Basketter et al. 1999	95.8	weak	-	OECD 2010
glycerol	180.16	90 %	Fluka	56-81-5	polyol / alcohol	non-binding	Patlewicz et al. 2008		No	-	Basketter et al. 1999	100.02	non	-	Natsch and Emter 2008
1,2-propanediol	76.09	99.5 %	Supelco	57-55-6	glycol / dihydric alcohol	non-binding	Patlewicz et al. 2008		No	-	Basketter et al. 1999	100.11	non	-	Natsch and Emter 2008
1-bromobutane	137.02	95 %	Sigma	109-65-9	alkyl halide	SN2	Patlewicz et al. 2008		No	-		NC	non	-	Gerberick et al. 2005
4-hydroxybenzoic acid	138.12	99 %	Aldrich	99-96-7	phenolic / benzoic acid derivative	non-binding	Patlewicz et al. 2008		No	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
4-methoxyacetophenone	150.18	99 %	Sigma-Aldrich	100-06-1	methyl keton	non-binding	Patlewicz et al. 2008		No	-	Gerberick et al. 2001	NC	non	-	Gerberick et al. 2005
6-methylcoumarin	149.59	99%	Sigma	92-48-8	lactone	Michael acceptor	Patlewicz et al. 2008		No	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
chlorobenzene	146.1	98.5 %	Fluka	108-90-7	aryl halide	non-binding	Patlewicz et al. 2008		Yes	-	OECD 2010	NC	non	-	Gerberick et al. 2005
DL-lactic acid	172.18	99.80%	Sigma	50-21-5	alpha-hydroxy carboxylic acid / alkanoic acid	non-binding	Patlewicz et al. 2008		Yes	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
fumaric acid	30.3	99.70%	Sigma	110-17-8	dicarbonic acid	non-binding	author's data		No	-	Hanson and Thoernehy-Andersen, 2003	NC	non	-	Kreiling et al. 2008
glucose	154.25	98 %	Aldrich	53-99-7	carbohydrate	non-binding	author's data		No	-	human metabolism (see methods)			-	human metabolism (see methods)
isopropanol	164.2	99 %	Fluka	67-63-0	alkanol / aliphatic secondary alcohol	non-binding	Patlewicz et al. 2008		Yes	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
methyl salicylate	152.1	99 %	Aldrich	119-36-8	benzoate (ester) / phenolic	non-binding	Patlewicz et al. 2008		Yes	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
n-butanol	74.12	99 %	Sigma-Aldrich	71-36-3	primary alkyl halide	non-binding	Patlewicz et al. 2008		No	-	Ryan et al. 2010	NC	non	-	Gerberick et al. 2005
n-hexane	86.18	99%	Sigma	110-54-3	alkane	non-binding	Patlewicz et al. 2008		No	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
nickel chloride	129.60	98.5 %	Fluka	7718-54-9	heavy metal salt	coordination bonds	author's data		Yes	+	Basketter et al. 1999	NC	non	-	OECD 2010
p-aminobenzoic acid	137.14	98 %	Fluka	150-13-0	arylamine / benzoic aid derivative	non-binding	Aptula, and Roberts, 2006		No	-	Basketter et al. 1999	NC	non	-	Basketter et al. 2008
propyl 4-hydroxybenzoate	180.2	99.80%	Sigma	94-13-3	benzoate (ester) // phenolic	non-binding	Patlewicz et al. 2008		No	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
salicylic acid	138.12	98 %	Sigma-Aldrich	69-72-7	benzoic acid derivative / phenolic	non-binding	Patlewicz et al. 2008		Yes	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
sulfanilamide	172.2	99%	Sigma	63-74-1	benzenesulfoneamide / arylamine	non-binding	Patlewicz et al. 2008		No	-	Basketter et al. 1999	NC	non	-	Gerberick et al. 2005
vanillin	152.15	98.5 %	T.J.Baker	121-33-5	phenolic aldehyde	non-binding	Patlewicz et al. 2008		No	-	Uter et al. 2007	NC	non	-	Gerberick et al. 2005
hexadecyltrimethylammonium bromide	79.1	99 %	Sigma	57-09-0	alkyl ammonium	non-binding	author's data		No	-	Andersen and Frankild, 1997			n.d.	no suitable data available

n.d.: no suitable data available

chemical structure able to react with proteins and, if yes, to identify possible mechanisms involved.

2.4. Direct Peptide Reactivity Assay (DPRA)

The DPRA was conducted using the synthetic lysine or cysteine model peptides and methods described by Gerberick and co-workers (Gerberick et al., 2004, 2007). In brief, the test substances were preferably dissolved in acetonitrile (Sigma–Aldrich, Germany) to prepare a 100 mM solution. If the test substances were not soluble in acetonitrile, solutions were prepared in water, methanol, propanol, isopropanol, acetone or mixture of these solvents which is in accordance with the DPRA protocol used in the interlaboratory ring trials. Substances were freshly prepared prior to use. The cysteine model peptide (Ac-RFAACAA-COOH) was prepared as 0.667 mM stock solutions in 100 mM phosphate buffer (100 mM NaH_2PO_4, 100 mM Na_2HPO_4, both Sigma–Aldrich, Germany, pH 7.5) and lysine model peptide (Ac-RFAAKAA-COOH) was prepared 100 mM ammonium acetate buffer (1.542 g ammonium acetate in 200 mL H_2O; pH 10.2; both peptides were obtained from Synbiosci, Livermore CA, USA). Peptide/test sample solutions were prepared in a ratio of 1:4:15 (v/v/v) substance:acetonitrile:cysteine peptide or in a ratio of 1:3 (v/v) substance:lysine peptide and then incubated in the dark for 24 h at room temperature and analyzed in triplicates for each peptide. Analysis of free peptides was performed by RP-HPLC (Agilent 1200 Series) with UV detection at 220 nm using a C18 Phenomex Luna® column (2.0 × 100 mm × 3 μm particle; Phenomex). Separation of peptides was performed by isocratic flow with a flow rate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 min, followed by an increase to 90% acetonitrile.

2.5. LuSens assay

The reporter gene cell line LuSens was prepared in collaboration with Dr. Christoph J. Wruck, RWTH Aachen University. This keratinocyte cell line derived from HaCaT cells carries a reporter gene for luciferase under the control of an antioxidant-response-element (ARE) and hence monitors Nrf2 transcription factor activity. The ARE promoter belongs to the NADPH:quinone oxidoreductase 1 gene from rats. The LuSens assay was performed similarly to that of the KeratinoSens™ assay and as follows: LuSens cells were cultured in complete DMEM culture medium with high glucose (PAA, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Germany), 100 U/mL penicillin – 100 μg/mL streptomycin (Biochrom AG, Germany), and 0.5 μg/mL puromycin (Sigma–Aldrich, Germany) in T75 culture flasks (TPP; Switzerland). Cells were grown for 24 h in 96-well plates prior to the substance treatment (1×10^4 cells in 120 μL medium). Stock solutions of the substances were prepared by dissolving in distilled water or in DMSO (final concentration of 200 mM) and diluted in 2-fold dilutions. Test substance solutions were further diluted in medium containing 1% FBS w/o puromycin to obtain a DMSO concentration of 1%. Treatment was performed by replacing regular cell culture medium with medium containing the test substance. For substances dissolved in water, the final DMSO concentration was adjusted to 1%. Each substance was tested in triplicates and in three independent experiments in dilutions ranging from 1 to 2000 μM. Cinnamic aldehyde (Sigma, Germany) was used as positive control in the concentration range of 2–32 μM. Substance incubation was performed under standard cell culture conditions for 48 h and luciferase activity then determined using SteadyGlo™ (Promega, Germany) according to manufacturer's instructions and the PerkinElmer Victor^3 photometer (PerkinElmer, Germany). Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay (Mosmann, 1983). Briefly, a 5 mg/mL MTT stock solution was prepared in phosphate buffered saline (PBS), and then diluted (1:10) in medium containing 1% FBS prior to use. This working solution (200 μL) was added to each well of the 96-well microtiter plate and incubated for further 2 h under standard cell culture conditions. For analysis medium was aspirated and cells were lysed by adding 100 μL of lysis solution (99.6 mL DMSO, Sigma–Aldrich, Germany; 10 g SDS, Sigma–Aldrich, Germany; and 0.4 mL glacial acetic acid, Merck, Germany). Absorbance was measured at 570 nm with reference wavelength at 690 nm using the PerkinElmer Victor^3 photometer.

2.6. KeratinoSens™ Assay

The KeratinoSens™ cell line was kindly provided by Andreas Natsch, Givaudan. This cell-line and the optimization of the protocol has been previously described in detail (Emter et al., 2010). The cell-line contains a stable insertion of a luciferase gene under the control of the ARE-element of the gene AKR1C2. The KeratinoSens™ assay was performed according to Givaudan's protocol (Emter et al., 2010). Briefly, KeratinoSens™ cells were cultured in complete DMEM culture medium with GlutaMAX™-1 (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany) and 0.55 mg/mL Geneticin (G-418; Invitrogen, Germany) in T75 culture flasks (TPP; Switzerland). For chemical exposure, cells were grown for 24 h in 96-well plates (1×10^4 cells in 120 μL medium). Prior to treatment, medium was aspirated and renewed by adding 150 μL medium containing 1% FBS w/o G-418. Stock solutions of the substances were prepared by diluting in distilled water or in DMSO (final concentration of 200 mM) and diluted in a common ratio of 1:2. Substances were further diluted in a 25-fold manner by adding 10 μL stock solution to 240 μL medium containing 1% FBS w/o G-418 resulting in 4× stock solution. Treatment was performed by adding 50 μL of each 4× stock solution. For chemicals dissolved in water the DMSO level was adjusted to 1%. Each compound was tested at dilutions ranging from 1 and 2000 μM as triplicates and repeated in three independent experiments. Cinnamic aldehyde was used as positive control in the concentration range of 4–64 μM. Substance incubation was performed under standard cell culture conditions for 48 h and luciferase activity then determined using SteadyGlo™ (Promega, Germany) according to manufacturer's instructions and the PerkinElmer Victor^3 photometer (PerkinElmer, Germany). Luciferase activity was then compared to the vehicle controls. Cytotoxicity was assessed using the MTT cytotoxicity assay. Briefly, 5 mg/mL stock solution was prepared in phosphate buffered saline (PBS), and then diluted (1:10) in medium containing 1% FBS prior to use. 200 μL of this working solution were added to each well of the 96-well microtiter plate and incubated for further 2 h. For analysis medium was aspirated and cells were lysed by adding 100 μL of lysis solution (99.6 mL DMSO, Sigma–Aldrich, Germany; 10 g SDS, Sigma–Aldrich, Germany; and 0.4 mL glacial acetic acid, Merck, Germany). Absorbance was measured at 570 nm with reference wavelength at 690 nm using the PerkinElmer Victor^3 photometer.

2.7. Modified myeloid U937 skin sensitization test (mMUSST)

The mMUSST assay uses the human histiocytic lymphoma cell line U937 (Ashikaga et al., 2006). The assay was performed as described previously (Bauch et al., 2011). Briefly, U937 cells were obtained from the German Resource Center for Biological Material (*DMSZ*; ACC 5). Cells were cultured in suspension as described using complete RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany) and 100 U/mL penicillin – 100 μg/mL streptomycin (Biochrom AG, Germany). For substance incubation, cells were seeded in 96-well microtiter plates (100 μL

of 0.5 × 10^6 cells/mL per well). Substances were dissolved either in medium (2× stock solution) or DMSO (400× stock solution). DMSO-solved substances were further diluted (1:200) in medium to obtain 2× stock solution. Final DMSO concentration in the test medium did not exceed 0.25%. Treatment was performed by addition of 100 μL of test substance to the cells. For each substance five concentrations were tested in duplicate. Concentrations were chosen based on preliminary propidium iodide (PI) cytotoxicity assays. To this accord, cells were exposed for 48 h to 12 concentrations obtained in a 2-fold dilution series starting at 2000 μg/mL for solid test substances or 1000 μg/mL for liquid test substances. Assessment of viable cells was performed as described previously (Python et al., 2007). The highest tested concentration in the main experiment was the two times the concentration causing a cytotoxicity of 25% (CV75). The additional concentrations were obtained in a 2-fold dilution series of the CV75. Each concentration was run in duplicate and each experiment was performed minimum in two independent experiments. Cells were exposed to the test substance for 48 h under standard cell culture conditions. After treatment, cells were centrifuged at 400*g* for 5 min at room temperature (RT) and washed once with PBS containing 5% FBS. Cells were resuspended in 100 μL PBS w/ 5% FBS and labeled for 30 min at 4 °C in the dark with 5 μL IgG-FITC (Cat. No. 555748, BD Pharmingen, Germany) or 5 μL anti-CD86-FITC antibody (Cat. No. 555657, BD Pharmingen, Germany). Following incubation, cells were washed twice with PBS w/ 5% FBS and resuspended in PBS. For cell viability analysis cell nuclei were stained with propidium iodide (PI; 50 μg/mL in PBS) for 5 min at RT in the dark. Analysis of the plasma membrane markers was performed in 10,000 living cells by flow cytometry using a BeckmanCoulter FC500MPL equipped with MXP software.

2.8. Human cell line activation test (h-CLAT)

The dendritic cell activation assay using the human monocytic leukemia cell line THP-1 was performed according to previous reports (Ashikaga et al., 2006, 2010; Sakaguchi et al., 2006, 2007) with minor modifications (Bauch et al., 2011). Briefly, THP-1 cells were obtained from the German Resource Center for Biological Material (DMSZ; ACC 16) and cultured in suspension as described in complete RPMI 1640 with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany), 100 U/mL penicillin, 100 μg/mL streptomycin (Biochrom AG, Germany) and 0.05 mM of 2-mercaptoethanol (Invitrogen, Germany). For experiments, cells were seeded in 24-well plates (TPP, Switzerland; 1 × 10^6 cells in 500 μL per well). Substances were dissolved in medium (2× stock solution) or DMSO (500× stock solution). DMSO-solved substances were further diluted (1:250) in medium to obtain 2× stock solution. Final DMSO concentration on the cells did not exceed 0.2%. Treatment was performed by applying 500 μL of the test substance dilution to each well for 24 h. Each substance was tested in eight concentrations in duplicate. Only if the classification in both tests differed, was a third test conducted. Concentrations were chosen according to preliminary PI cytotoxicity assays. To this accord, cells were exposed to 12 concentrations obtained in a 2-fold dilution series starting at 2000 μg/mL for solid test substances or 1000 μg/mL for liquid test substances. Assessment of viable cells was performed as described previously (Ashikaga et al., 2010) (Bauch et al., 2011). The highest tested concentration in the main experiment was 1.2 × CV75. The additional concentrations were obtained by a 1:1.2 dilution series of the 1.2 × CV75. Each concentration was run in duplicate and each experiment was performed minimum in two independent runs. For analysis cells were transferred into 1.5 mL tubes (Eppendorf, Germany). Cells were centrifuged at 200*g* and 4 °C for 5 min and washed twice with 1 mL staining buffer, PBS w/Ca^{2+} Mg^{2+} (Biochrom AG, Germany) and 0.1% BSA (Sigma, Germany. Cells were resuspended in 600 μL staining buffer containing 0.01% Cohn fraction (Sigma, Germany) and incubated for 15 min at 4 °C in the dark (original protocol: 10 min incubation). After incubation, cells were distributed in V-shaped 96-well microtiter plate (TPP, Switzerland). Each concentration replicate was split into 3 wells by pipetting 180 μL cell suspension per well. Cells were centrifuged at 200*g* and 4 °C for 5 min and resuspended in 50 μL antibody working solution. Cells were stained for IgG1-FITC (1:16 diluted in PBS; DAKO/DAK X092701) and anti-CD54-FITC (1:16 diluted in PBS; DAKO /DAKO F7143) or anti-CD86-FITC (1:7 diluted in PBS; Cat. No.: 555657, BD Pharmingen, Germany). Staining was performed at 4 °C in the dark for 30 min. After incubation, cells were washed twice in 200 μL staining buffer; centrifugation was performed at 200*g* and 4 °C for 5 min. Cells were resuspended in 200 μL staining buffer. For DNA staining, 5 μL PI solution (50 μg/mL in PBS) was added for 5 min in the dark before flow cytometric analysis (original protocol: 1.2 μg/mL). Flow cytometry was performed with a Beckman Coulter FC500MPL and the MXP software.

2.9. Data evaluation and statistics

Data analysis was performed in MS Excel. The peptide depletion in the DPRA was determined by dividing the areas under the curves (AUC) of peptide (treated and untreated) by the AUC of peptide incubations with the vehicle alone. Mean peptide depletion was calculated using following equation: mean peptide depletion = [(AUC T.C. (Cys-peptide)/(AUC V.C. (Cys-peptide) + (AUC T.C. (Lys-peptide)/(AUC V.C. (Lys-peptide)/2∗100. For the LuSens and KeratinoSens™ assays, those concentrations inducing less than 70% of cell viability were excluded from further analysis. The fold induction of the luminescent signal was calculated by dividing the relative luminescence units (RLU) of the treated cells by the RLU of control cells using following equation: $FI = (RLU_{T.C.})/(RLU_{C.C.})$. For the mMUSST assays, concentrations inducing viability less than 70% were not considered for further assessment of the sensitization potential, whereas for the h-CLAT a cell viability threshold of 50% was used. The mMUSST assay was evaluated by setting the isotype control of medium or vehicle treated cells to 0.6% and fold induction was calculated by dividing the value of substance treated cells by the value of control cells using the following formula: $FI = (\%CD86_{T.C.} - \%isotype_{T.C.})/(\%CD86_{C.C.} - \%isotype_{C.C.})$. The h-CLAT assay was evaluated using the mean fluorescence intensity (MFI) and fold induction was calculated by dividing the MFI of substance treated cells by the MFI of non-treated cells using following equation for CD86, $FI\ (CD86) = (MFI\ CD86_{T.C.} - MFI\ isotype_{T.C.})/(MFI\ CD86_{C.C.} - MFI\ isotype_{C.C.})$, or CD54, $FI\ (CD54) = (MFI\ CD54_{T.C.} - MFI\ isotype_{T.C.})/(MFI\ CD54_{C.C.} - MFI\ isotype_{C.C.})$, calculations.

Substances that induced mean peptide depletion of cysteine- and lysine-containing peptide above 6.4% were rated as being peptide reactive, while in the LuSens and KeratinoSens™ assay, those substances inducing greater luciferase expression than 1.5-fold induction were considered to have an ARE activating potential. In the DC-like activation assays, those substances that induced the expression of CD86 higher than 1.2-fold compared to the controls in mMUSST assay were predicted to have a DC activating potential, while in the h-CLAT assay substances inducing a fold induction greater than 2.0-fold for CD54 and/or 1.5-fold increase for CD86 were predicted to have a DC activating potential in at least 2 of 3 experiments (new prediction model as described in Sakaguchi et al. (2010); original protocol: CD54 considered to be positive if >1.5) . For the purpose of predicting the sensitization potential of a substance, if the above mentioned thresholds are exceeded, the substance would be categorized as being a skin sensitizer. Statisti-

Table 2
Results of peptide reactivity assessments: OECD QSAR Toolbox v2.0 (*in silico*), the direct peptide reactivity assay (DPRA; *in chemico*), and the LuSens assay and KeratinoSens™ assay (both *in vitro*; ARE-reporter gene based assay).

Substance	DPRA			OECD Toolbox v2.0		LuSens	KeratinoSens™
	Lys-peptide depletion	Cys-peptide depletion	Mean peptide depletion	Rationale by OECD toolbox	Prediction	EC1.5 in μM	EC1.5 in μM
Oxazolone	44.6	71.7	58.1	Nucleophilic acyl substitution	+	220	155
MCI/MI	4.3	90.9	47.6	Ring opening at the S–N bond followed by Nucleophilic addition.	+	3	2
p-Benzoquinone	95.9	99.7	97.8	Michael addition on Quinones/quinone imines	+	4	6
1-Chloro-2,4-dinitrobenzene	58.3	99.9	79.1	Nucleophilic substitution on activated aryl carbon atom.	+	2	3
4-Phenylenediamine	42.2	99.8	71.0	No binding	–	6	5
Propyl gallate	63.6	59.2	61.4	No binding	–	6	191
2,4,6-Trinitrobenzenesulfonic acid	22.6	99.7	61.1	No binding	–	23	41
Phthalic anhydride	31.3	16.7	24.0	Protein acylation by acid anhydride	+	Below threshold	Below threshold
Formaldehyde > 36% (1% DMSO)	2.4	54.8	28.6	Schiff base formation with aldehydes.	+	101	137
Methyldibromo glutaronitrile	28.4	75.3	51.8	Nucleophilic substitution on halogenated C sp^3 atom	+	7	8
Isoeugenol	21.8	99.1	60.4	No binding	–	3	6
Diethyl maleate	78.8	99.8	89.3	Michael addition on conjugated systems with electron withdrawing groups	+	4	2
Ethylene diamine	0.7	18.6	9.7	No binding	–	Below threshold	549
Benzylidene acetone	−1.4	92.4	45.5	Michael addition on conjugated systems with electron withdrawing groups	+	7	7
Cobalt chloride	35.0	30.3	32.6	No binding	–	75	106
2-Phenylpropionaldehyde	5.1	26.6	15.8	Schiff base formation with aldehydes	+	34	26
α-Hexyl-cinnamic aldehyde	−0.9	12.3	5.7	Michael addition on α,β-aldehydes, Schiff base formation with aldehydes.	+	13	23
Tartaric acid	−2.0	6.6	2.3	No binding	–	Below threshold	3
2-Mercaptobenzothiazole	3.2	99.9	51.5	Protein thiol-disulfide interchange	+	85	57
2,3-Butanedione	34.9	92.8	63.9	Nucleophilic cycloaddition to diketones	+	117	74
Citral	8.6	78.6	43.6	Michael addition on α,β-aldehydes, Schiff base formation with aldehydes.	+	6	17
Eugenol	4.2	38.3	21.3	No binding	–	79	64
Farnesal	−4.1	37.3	16.6	Michael addition on α,β-aldehydes, Schiff base formation with aldehydes.	+	6	11
Sodium lauryl sulfate	97.1	−0.1	48.5	No binding	–	Below threshold	Below threshold
4-Allylanisole	−2.2	98.5	48.1	No binding	–	274	39
Hydroxycitronellal	32.8	61.7	47.2	Schiff base formation with aldehydes	+	185	31
Phenyl benzoate	2.2	64.9	33.6	Diarylester aminolysis or thiolysis	+	Below threshold	Below threshold
Cinnamic alcohol	2.7	21.3	12.0	No binding	–	20	24
Imidazolidinyl urea	20.6	59.0	39.8	Protein acylation by *N*-acylamides	+	80	41
Undecylenic acid	−0.1	11.4	5.7	No binding	–	27	57
Ethylene glycol dimethacrylate	33.9	93.5	63.7	Michael addition on conjugated systems with electron withdrawing groups	+	18	14
Pyridin	−2.2	−0.1	−1.2	No binding	–	Below threshold	Below threshold
Aniline	2.4	−1.0	0.7	No binding	–	165	2
Methyl methacrylate	11.8	55.3	33.5	Michael addition on conjugated systems with electron withdrawing groups	+	Below threshold	Below threshold
Xylene	0.7	−0.7	0.0	No binding	–	Below threshold	Below threshold
Glycerol	1.3	−0.7	0.3	No binding	–	Below threshold	Below threshold
1.2-Propanediol	0.0	−3.0	−1.5	No binding	–	Below threshold	Below threshold
4-Hydroxybenzoic acid	0.7	3.0	1.9	No binding	–	Below threshold	Below threshold
4-Methoxyacetophenone	−0.3	−1.9	−1.1	No binding	–	168	56
6-Methylcoumarin	−2.0	−1.8	−1.9	No binding	–	14	6
DL-lactic acid	−1.2	3.0	0.9	No binding	–	Below threshold	Below threshold
Fumaric acid	4.6	10.8	7.7	No binding	–	Below threshold	Below threshold
Glucose	15.3	0.1	7.7	Schiff base formation with aldehydes	–	Below threshold	Below threshold
Isopropanol	−1.7	−2.0	−1.9	No binding	–	Below threshold	Below threshold

(*continued on next page*)

Table 2 (*continued*)

Substance	DPRA			OECD Toolbox v2.0		LuSens	KeratinoSens™
	Lys-peptide depletion	Cys-peptide depletion	Mean peptide depletion	Rationale by OECD toolbox	Prediction	EC1.5 in μM	EC1.5 in μM
Methyl salicylate	−0.2	−2.4	−1.3	No binding	–	383	Below threshold
n-Butanol	−1.3	0.7	−0.3	No binding	–	Below threshold	Below threshold
n-Hexane	2.6	−0.9	0.9	No binding	–	Below threshold	562
Nickel chloride	0.3	43.1	21.7	No binding	–	355	Below threshold
p-Aminobenzoic acid	0.5	5.9	3.2	No binding	–	Below threshold	Below threshold
Propyl 4-hydroxybenzoate	−1.3	−1.5	−1.4	No binding	–	9	5
Salicylic acid	1.0	8.7	4.8	No binding	–	Below threshold	Below threshold
Sulfanilamide	−10.4	3.8	−3.3	No binding	–	Below threshold	Below threshold
Vanillin	19.8	−1.3	9.3	No binding	–	226	83
Hexadecyltrimethylammonium bromide	1.5	2.4	2.0	No binding	–	Below threshold	Below threshold

cal significance was assessed using a t-test. The predictivity of single assays or combinations was calculated according to Cooper statistics (Cooper et al., 1979) using the following to assess sensitivity, specificity, positive and negative predictive value and accuracy: Sensitivity: (RP/[RP + FN] × 100); Specificity: (FP/[RN + FP] × 100); Positive predictivity: (RP/[RP + FP] × 100); Negative predictivity: ((RN/[RN + FN] × 100); Accuracy: (RP + RN/[RN + RP + FP + FN] × 100).

3. Results

In this study, comparative testing using the DPRA, LuSens, KeratinoSens™, mMUSST and h-CLAT assays was performed to establish (LuSens) or expand (DPRA, KeratinoSens™, mMUSST and h-CLAT) the available database for the test methods. The results obtained from these tests along with the predictions obtained from the OECD QSAR Toolbox Version 2.0 are depicted in Tables 2 and 3. The predictivity of the individual methods and their combinations were compared to human and LLNA data using Cooper statistics (Table 4). The results were then used to propose first approaches for developing testing strategies to assess skin sensitization using non-animal alternatives. Of the 59 test substances initially selected, 54 could be assessed using the chosen test systems whereas five substances where excluded due to technical limitations (see discussion). An overview of the test results is given in Table 5.

3.1. Local lymph node assay (LLNA)

In the murine LLNA (Basketter et al., 1999b; Kimber et al., 2003) one substance was assessed as being a false negative, i.e. nickel chloride; and four substances were assessed as being false positive: pyridine, sodium dodecyl sulfate (SDS), tartaric acid and xylene if compared to human data. Based on these results, the LLNA offered a sensitivity of 96% and a specificity of 81% whereas the overall accuracy was 90%.

3.2. Reaction mechanism based analysis – OECD QSAR Toolbox and DPRA

The protein reactivity of test substances can be estimated *in silico* via the correlation of their chemical structures with structural alerts. The predictive capacity of the OECD QSAR Toolbox Version 2.0 was assessed and used to discern the potential chemical reactivity involved. The substances were processed both with and without the module of skin metabolism simulation. The results obtained without skin metabolism by this analysis are shown in Table 2. The OECD QSAR Toolbox Version 2.0 was able to correctly predict 40 of 50 or 37 of 53 of the substances when compared to the human data or LLNA data, respectively, whereby in particular the predictions of pro- and prehaptens proved to be difficult. Inclusion of the skin metabolism module identified several protein-binding and non-binding metabolites both for the known pro- or prehaptens and but also for the known nonsensitizing substances (data not shown). For example, when analyzing the skin sensitizer propyl gallate, nine metabolites with four alerts for protein binding were predicted and for the irritant pyridine nine metabolites and two alerts for protein binding were predicted. Only predictions obtained without the metabolism module were evaluated. Evaluation of data with Cooper statistics (Cooper et al., 1979) revealed that the *in silico* method using the OECD QSAR Toolbox Version 2.0, offered a sensitivity of 64% or 56%, a specificity of 100% or 100% and an overall accuracy was 80% or 70% when comparing the predictions to human data or the LLNA, respectively (Table 4).

The DPRA is a method which simulates chemical protein-reactivity in an *in chemico* assay using two synthetic model peptides and specifically assesses the peptide reactivity to cysteine or lysine residues. The DPRA was able to correctly predict 43 of 50 or 42 of 53 of the substances when compared to human data or LLNA data, respectively. Three substances were rated as false negative: aniline, undecylenic acid and α-hexyl cinnamic aldehyde, whereas four substances were considered to be false positive: fumaric acid, glucose, SDS and vanillin (Table 2). The *in chemico* method offered a sensitivity of 89% or 81%, a specificity of 82% or 76% and an overall accuracy of 86% or 79% when compared to human data or the LLNA, respectively (Table 4).

3.3. ARE/Nrf 2 based reporter gene assays –LuSens and KeratinoSens™

The LuSens assay and the KeratinoSens™, both based on luciferase reporter cell lines and ARE pathway activation, can also be used to indirectly assess the intracellular cysteine reactivity of a substance and the resulting activation of the Keap-1/Nrf2 signaling pathway. The LuSens assay correctly predicted 42 out of 50 or 41 out of 53 substances when compared to human or LLNA data, respectively. Three substances exhibited borderline activation,

hexadecyltrimethylammonium bromide, hydroxycitronellal, methylmethacrylate since no dose dependent effect or high variability between experiments was observed. Three substances would be incorrectly rated to be negative: ethylene diamine, phenyl benzoate, phthalic anhydride; and five substances would be incorrectly rated to be positive: 4-methoxyacetophenone, 6-methylcoumarin, methyl salicylate, propyl-4-hydroxybenzoate, and vanillin (Table 2). For the LuSens assay, the Cooper statistics estimated a sensitivity of 89% or 81%; a specificity of 77% or 71% and an overall accuracy of 84% or 77% when compared to the human data or LLNA, respectively (Table 4). The KeratinoSens™ assay correctly predicted 40 out of 50 or 43 out of 53 substances when compared to human or LLNA data, respectively. Four substances would be incorrectly rated to be negative: methyl methacrylate, nickel chloride, phenyl benzoate, phthalic anhydride; and six substances would be incorrectly rated to be positive: 4-methoxyacetophenone, 6-methylcoumarin, *n*-hexane, propyl-4-hydroxybenzoate, tartaric acid and vanillin (Table 2). For the KeratinoSens™ assay, the Cooper statistics estimated a sensitivity of 86% or 83%; a specificity of 73% or 76% and an overall accuracy of 80% or 81% when compared to the human data or LLNA, respectively (Table 4).

3.4. Dendritic cell activation assays – mMUSST and h-CLAT

The dendritic cell activation assay mMUSST measures the expression of the cell surface protein CD86 as marker for maturation of dendritic-like cells induced by the test substance. The mMUSST assay was able to correctly predict 43 out of 50 or 39 of 53 substances when compared to human or LLNA data, respec-

Table 3
Summary results of DC-like cell activation assays: mMUSST and h-CLAT

Substance	mMUSST-CD86		h-CLAT-CD86		h-CLAT -CD54	
	EC1.2 in μg/mL	EC1.2 in μM	EC1.5 in μg/mL	EC1.5 in μM	EC2 in μg/mL	EC2 in μM
Oxazolone	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
MCI/MI	0	2	2	14	Below threshold	Below threshold
p-Benzoquinone	1	14	4	36	Below threshold	Below threshold
1-Chloro-2,4-dinitrobenzene	0	2	2	12	3	13
4-Phenylenediamine	1	5	2	14	Below threshold	Below threshold
Propyl gallate	2	8	Below threshold	Below threshold	10	48
2,4,6-Trinitrobenzenesulfonic acid	15	51	Below threshold	Below threshold	21	71
Phthalic anhydride	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Formaldehyde >36% (1% in DMSO)	1	21	Below threshold	Below threshold	Below threshold	Below threshold
Methyldibromo glutaronitrile	Below threshold	Below threshold	10	39	Below threshold	Below threshold
Isoeugenol	Below threshold	Below threshold	17	104	Below threshold	Below threshold
Diethyl maleate	13	75	62	358	Below threshold	Below threshold
Ethylene diamine	25	412	109	1818	Below threshold	Below threshold
Benzylidene acetone	4	26	17	115	Below threshold	Below threshold
Cobalt chloride	9	73	29	223	45	346
2-Phenylpropionaldehyde	Below threshold	Below threshold	28	209	Below threshold	Below threshold
α-Hexyl-cinnamic aldehyde	Below threshold	Below threshold	Below threshold	Below threshold	21	98
Tartaric acid	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
2-Mercaptobenzothiazole	7	44	Below threshold	Below threshold	Below threshold	Below threshold
2,3-Butanedione	13	153	27	319	Below threshold	Below threshold
Citral	Below threshold	Below threshold	13	87	13	86
Eugenol	6	35	77	469	90	548
Farnesal	Below threshold	Below threshold	Below threshold	Below threshold	9	40
Sodium lauryl sulfate	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
4-Allylanisole	110	744	315	2125	76	511
Hydroxycitronellal	4	22	22	126	Below threshold	Below threshold
Phenyl benzoate	12	63	82	416	Below threshold	Below threshold
Cinnamic alcohol	Below threshold	Below threshold	73	547	97	724
Imidazolidinyl urea	8	20	Below threshold	Below threshold	Below threshold	Below threshold
Undecylenic acid	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Ethylene glycol dimethacrylate	6	31	427	2156	287	1447
Pyridine	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Aniline	71	762	280	3007	Below threshold	Below threshold
Methyl methacrylate	1	5	198	1978	298	2978
Xylene	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Glycerol	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
1,2-Propanediol	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
4-Hydroxybenzoic acid	Below threshold	Below threshold	Below threshold	Below threshold	1052	7617
4-Methoxyacetophenone	Below threshold	Below threshold	70	469	Below threshold	Below threshold
6-Methylcoumarin	Below threshold	Below threshold	36	222	Below threshold	Below threshold
DL-lactic acid	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Fumaric acid	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Glucose	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Isopropanol	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Methyl salicylate	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
n-Butanol	Below threshold	Below threshold	921	12429	1066	14382
n-Hexane	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Nickel chloride	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
p-Aminobenzoic acid	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Propyl 4-hydroxybenzoate	Below threshold	Below threshold	Below threshold	Below threshold	83	461
Salicylic acid	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Sulfanilamide	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Vanillin	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold
Hexadecyltrimethylammonium bromide	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold	Below threshold

Table 4
Summary of predictivities of single assays and their combinations based on Cooper statistics.

Compared to human		Sensitivity (%)	Specificity (%)	Positive predictivity (%)	Negative predictivity (%)	Accuracy (%)
LLNA		96	81	87	94	90
single assays	OECD QSAR toolbox	64	100	100	69	80
	DPRA	89	82	86	86	86
	LuSens	89	77	83	85	84
	KeratinoSens™	86	73	80	80	80
	mMUSST	75	100	100	76	86
	h-CLAT	75	77	81	71	76
Combinations	DPRA and LuSens	100	64	78	100	84
	DPRA and KeratinoSens™	100	59	76	100	82
	DPRA and mMUSST	96	82	87	95	90
	DPRA and h-CLAT	96	59	75	93	80
	LuSens and mMUSST	96	77	84	94	88
	LuSens and h-CLAT	96	68	79	94	84
	KeratinoSens™ and mMUSST	97	56	78	91	81
	KeratinoSens™ and h-CLAT	93	64	76	88	80
Prediction Model (high overall accuracy)	DPRA, LuSens and mMUSST	93	95	96	91	94
	DPRA, KeratinoSens™ and mMUSST	93	95	96	91	94
compared to LLNA	Sensitivity	Specificity	PPV	NPV	Accuracy	
single assays	OECD QSAR toolbox	56	100	100	52	70
	DPRA	81	76	88	65	79
	LuSens	81	71	85	63	77
	KeratinoSens™	83	76	88	68	81
	mMUSST	64	94	96	55	74
	h-CLAT	72	76	87	57	74
combinations	DPRA and LuSens	92	59	83	77	81
	DPRA and KeratinoSens™	94	59	83	83	83
	DPRA and mMUSST	86	76	89	72	83
	DPRA and h-CLAT	89	53	80	69	77
	LuSens and mMUSST	86	71	86	71	81
	LuSens and h-CLAT	86	59	82	67	77
	KeratinoSens™ and mMUSST	89	71	86	75	83
	KeratinoSens™ and h-CLAT	89	65	84	73	81
Prediction Model (high overall accuracy)	DPRA, LuSens and mMUSST	81	88	94	68	83
	DPRA, KeratinoSens™ and mMUSST	81	88	94	68	83

tively. The substances citral, imidazolidinyl urea, nickel chloride, diethyl maleate and undecylenic acid exhibited borderline activation. Compared to human data seven substances would be identified as false negative: 2-phenylpropianaldehyde, cinnamic alcohol, isoeugenol, methyldibromo glutaronitrile, oxazolone, phthalic anhydride and α-hexyl cinnamic aldehyde. No false positive result was obtained with the mMUSST whereby if compared to LLNA one substance would be assessed as being false positive (nickel chloride), and 13 substances as false negative. The results are summarized in Table 3. The mMUSST assay offered a sensitivity of 75% or 64%, a specificity of 100% or 94%, and an overall accuracy of 86% or 74% when compared to human data or the LLNA, respectively (Table 4).

The dendritic cell activation assay h-CLAT measures the expression of the cell surface proteins CD86 and CD54 as markers for maturation of dendritic cells induced by the test substance. The h-CLAT assay was able to correctly predict 38 out of 50 or 39 out of 53 substances when compared to human or LLNA data, respectively. The substances 4-phenylenediamine, aniline, farnesal, p-benzoquinone, diethyl maleate, eugenol, salicylic acid, phthalic anhydride, propyl gallate and vanillin gave borderline results. Seven substances would be rated as negative but were actually skin sensitizers according to the human data: 2-mercaptobenzothizole, formaldehyde, nickel chloride, imidazolidinyl urea, oxazolone, phthalic anhydride and undecylenic acid. Whereas five substances would be rated as false positive: 4-hydroxybenzoic acid, 4-methoxyacetophenone, 6-methylcoumarin, *n*-butanol and propyl-4-hydroxybenzoate. The summary of results is given in Table 3. Cooper statistics revealed that the h-CLAT assay has a sensitivity of 75% or 72%, a specificity of 77% or 76%, and an overall accuracy of 76% or 74% when compared to human data or the LLNA, respectively (Table 4).

3.5. Incorrect predictions

Despite the generally good predictivities of the single methods, some substances were predicted either false positive or false negative. Table 5 summarizes the results of each single assay. Phthalic anhydride, a respiratory allergen, was false negative in three assays, whereas seven substances generated either false negative (oxazolone, α-hexyl cinnamic aldehyde, undecylenic acid) or false positive result (4-methoxyacetophenone, 6-methylcoumarin, propoyl-4-hydroxy benzoic acid and vanillin) in two of the assays. Further 19 substances resulted in a false negative (formaldehyde, methyldibromo glutaronitril, isoeugenol, ethylene diamine, 2-phenylpropionaldehyde, 2-mercaptobenzothiazole, farnesal, phenyl benzoate, cinnamic alcohol, imidazolidinyl urea, aniline, methyl methacrylate and nickel chloride) or false positive (SDS, 4-hydroxybenzoic acid, fumaric acid, glucose, methyl salicylate and *n*-butanol) in at least one of the four assays.

3.6. Prediction model

Based on the intended purpose of a specific test, different prediction models can be applied to the results of this study: Predictions can strive for high sensitivity (to minimize false negatives),

Table 5
Overview of the assays, their results and the results of the proposed prediction model.

													prediction model				
Substance	reaction mechanism	pro/prehapten	Human	EC3 (%)	potency	LLNA	OECD toolbox	DPRA	LuSens	Keratino Sens™	mMUSST	h-CLAT	DPRA + LuSens* (both negative then nonsensitizer)	mMUSST (if positive then sensitizer)	initial prediction	WoE (2 of 3)	Prediction
oxazolone	Acyl-transfer		+	0.003	extreme	+	+	+	−	+	−	−			?	+	+
MCI MI	Michael		+	0.01	extreme	+	+	+	−	+	+	+		+	+		+
p-benzoquinone	Michael		+	0.01	extreme	+	+	+	−	+	+	+		+	+		+
1-chloro-2,4-dinitrebenzene	SNAr		+	0.05	extreme	+	+	+	−	+	+	+		+	+		+
4-phenylenediamine	Pre-Michael	pre	+	0.16	strong	−	−	+	−	+	+	+		+	+		+
propyl gallate	Acyl-transfer	pre	+	0.32	strong	−	−	+	−	+	+	+		+	+		+
2,4,6-trinitrobenzenesulfonic acid	SNAr			0.36	strong	−	−	+	−	+	+	+		+	+		+
phthalic anhydride	Acyl-transfer		+	0.36	strong	−	+	+	-	-	-	-			?	-	-
formaldehyde >36%	Schiff base		+	0.61	strong	−	+	+	−	+	+	-		+	+		+
methyldibromo glutaronitrile	Pre-Michael		+	0.96	strong	−	+	+	−	+	-	+			?	+	+
isoeugenol	Pre-Michael	pre pre	+	1.56	moderate	−	-	+	−	+	-	+			?	+	+
diethyl maleate	Michael		+	2.16	moderate	−	+	+	−	+	+	+		+	+		+
ethylene diamine	pro-Schiff base	pre	!	2.26	moderate		-	!	-	!	!	!		+	+		+
benzylidene acetone	Michael		!	3.76	moderate		!	!		!	!	!		+	+		+
cobalt chloride	coordination bonds		!	4.86	moderate		-	!		!	!	!		+	+		+
2-phenylpropionaldehyde	SchiTbase		+	5.91	moderate	−	+	+	−	+	-	+			?	+	+
α-hexyl-cinnamic aldehyde	Michael		+	8.00	moderate	−	+	-	−	+	-	+			-		-
tartaric acid	non-binding		-	8.70	moderate	−	-	-	-	+	-	-	-		-		-
2-mercaptobenzothiazole	Acyl-transfer		+	9.70	moderate	−	+	+	−	+	+	-		+	+		+
2,3-butanedione	Schiff base			11.00	weak	−	+	+	−	+	+	+		+	+		+
citral	Schiff base		+	13.00	weak	−	+	+	−	+	-	+		+	+		+
eugenol	Pre-Michael	pro	+	13.00	weak	−	-	+	−	+	+	+		+	+		+
famegal	Schiff base			13.00	weak	−	+	+	−	+	-	+			?	+	+
sodium lauryl sulfate	non-binding		-	14.00	weak	−	-	+	-	-	-	-			?	-	-
4-allylanisole	Pre-Michael	pro		18.00	weak	−	-	+	−	+	+	+		+	+		+
hydroxycitronellal	Schiff base		+	20.00	weak	−	+	+	−	+	+	+		+	+		+
phenyl benzoate	Acyl-transfer		+	20.00	weak	−	+	+	-	-	+	+		+	+		+
cinnamic alcohol	Pre-Michael	pro	+	21.00	weak	−	-	+	−	+	-	+			?	+	+
imidazolidinyl urea	Acyl-transfer		+	24.00	weak	−	+	+	−	+	+	-		+	+		+
undecylenic acid	non-binding		+	25.00	weak	−	-	-	−	+	-	-		+	+		+
ethylene glycol dimethacrylate	Michael		!	35.00	weak		!	!		!	!	!			+		+
pyridin	non-binding		-	71.20	weak		-	-	-	-	-	-	-		-		-
aniline	SNAr	pro	!	89.00	weak		-	-		!	!	!		+	+		+
methyl methacrylate	Michael		+	90.00	weak	−	+	+	-	-	+	+		+	+		+
xylene	non-binding		-	95.8	weak	−	-	-	-	-	-	-	-		-		-
glycerol	non-binding		-	100.62	non	-	-	-	-	-	-	-	-		-		-
1,2-propanediol	non-binding		-	100.11	non	-	-	-	-	-	-	-	-		-		-
4-hydroxybenzoic acid	non-binding		-	NC	non	-	-	-	-	-	-	+	-		-		-
4-methoxyacetophenone	non-binding		-	NC	non	-	-	-	−	+	-	+			-		-
6-methylcoumarin	Michael		-	NC	non	-	-	-	−	+	-	+			-		-
DL-lactic acid	non-binding		-	NC	non	-	-	-	-	-	-	-	-		-		-
fumaric acid	non-binding		-	NC	non	-	-	+	-	-	-	-			?	-	-
glucose	non-binding		-	NC	non	-	-	+	-	-	-	-			?	-	-
isopropanol	non-binding		-	NC	non	-	-	-	-	-	-	-	-		-		-
methyl salicylate	non-binding		-	NC	non	-	-	-	−	-	-	-			-		-
n-butanol	non-binding		-	NC	non	-	-	-	-	-	-	+	-		-		-
n-hexane	non-binding		-	NC	non	-	-	-	-	+	-	-	-		-		-
nickel chloride	coordination bonds		+	NC	non	-	-	+	−	-	-	-		+	+		+
p-aminobenzoic acid	non-binding		-	NC	non	-	-	-	-	-	-	-	-		-		-
propyl 4-hydroxybenzoate	non-binding		-	NC	non	-	-	-		!	-	!			-		-
salicylic acid	non-binding		-	NC	non	-	-	-	-	-	-	-	-		-		-
sulfanilamide	non-binding		-	NC	non	-	-	-	-	-	-	-	-		-		-
vanillin	non-binding		-	NC	non	-	-	+	−	+	-	-			?	+	+
hexadecyltrimethylammonium bromide	non-binding		-				-	-	-	-	-	-	-		-		-

* LuSens can be replaced by KeratinoSens™

high specificity (to minimize false positives) or a high overall accuracy.

The DPRA and LuSens assays simulate an essential early step, namely protein reactivity, in the pathway leading to the adverse outcome of skin sensitization. The LuSens assay also yields additional information on the activation of the ARE related genes resulting from the subsequent activation of Keap-1/Nrf2 signaling pathway. The combination of both peptide reactivity assays, DPRA and LuSens (the KeratinoSens™ assay can be used instead of the LuSens assay), is proposed. Based on the results from this study, if the criterion "one assay results in a positive response" is used, 100% of the human skin sensitizers in the substance panel were identified (high sensitivity). Yet, this criterion would also result in eight of 22 nonsensitizers being predicted as positive. Hence it does not allow for reliable identification of skin sensitizers (low specificity; a positive result does not yield information with sufficient predictivity). However, if both assays indicate no protein reactivity, a sensitizing potential can be excluded with high probability. Based on this result, protein reactivity is a necessary, but not a sufficient prerequisite for skin sensitization. Conversely, the mMUSST did not yield a positive response for any human nonsensitizers (high specificity). Yet, this assay predicted seven human skin sensitizers to be negative. Hence it does not allow for reliable identification of nonsensitizers (low sensitivity; a negative result does not yield information with a sufficient predictivity). However, if this assay indicates DC activation, a sensitizing potential can be confirmed with high probability. Based on this result, DC activation is a sufficient, but not a necessary prerequisite for skin sensitization. Yet on occasion, protein reactivity and DC-activation will give inconclusive results (protein reactivity positive and mMUSST negative) or conflicting results (protein reactivity negative and mMUSST positive).

The model proposed is depicted in Fig. 1. It combines the sensitivities and specificities described. If the two peptide-reactivity assays, DPRA and LuSens, yield negative results, then the substance is considered to be nonsensitizing. If substances are predicted to be sensitizing in the mMUSST assay, the substance is considered to be a skin sensitizer. If the predictions are conflicting, or if the h-CLAT is used instead of the mMUSST, a pragmatic 'weight of evidence' approach is used: Any two of the three tests rule the overall result (any two assays must be positive to rate the substance as a skin sensitizer and any two assays must be negative to rate the substance as a nonsensitizer). Using this model, 47 out of 50 or 44 out of 53 test substances were predicted correctly if compared to human or LLNA, respectively. One substance would be rated as false positive (vanillin) and two substances would be rated as false negative (phthalic anhydride and α-hexyl cinnamic aldehyde) if compared to human data; comparison to data from the LLNA would result in two false positives (nickel chloride and vanillin) and seven false negatives (6-methylcoumarin, phthalic anhydride, pyridin, SDS, tartaric acid, xylene and α-hexyl cinnamic aldehyde). Therefore, the predictivities of this prediction model are as follows: sensitivity of 93% or 81%, specificity of 95% or 88% and an overall accuracy of 94% or 83% if compared to human data or LLNA, respectively (Table 4).

To use this prediction model for classification purposes, all three tests need to be performed. If specific questions regarding the sensitization potential are asked (e.g. the reliable exclusion of a sensitization potential for screening purposes) testing of effect, protein reactivity or DC activation, may be sufficient.

4. Discussion

The development of animal-free test methods to assess the skin sensitizing potential of substances (e.g. chemicals and cosmetic ingredients) has been the goal of many researchers for some time. Much of this is being driven by the European legislation, in particular the Cosmetics Directive (Council Directive 76/768/EEC) and its successor the Cosmetics Regulation (Council Directive 76/768/EEC, 1976; EC, 2009). A number of *in vitro* assays for sensitization testing have been developed and are currently in the validation process at ECVAM, namely the DPRA, h-CLAT, MUSST and KeratinoSens™ assay. Moreover, scientists are also aware of the

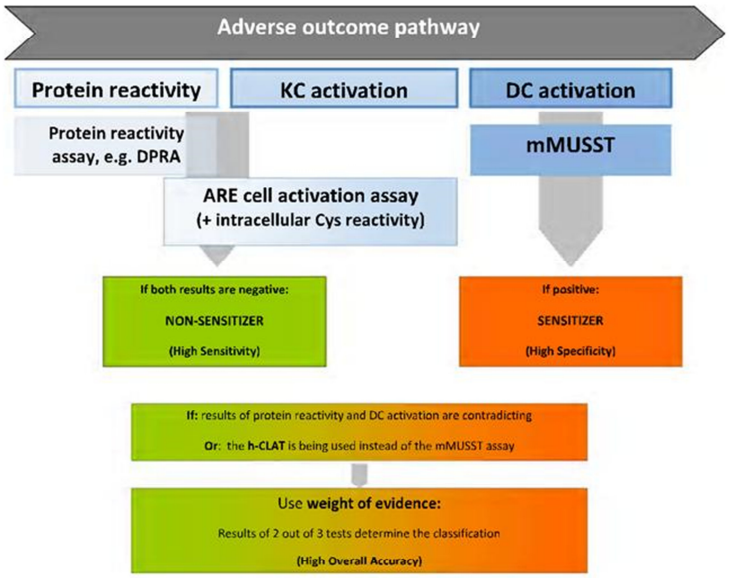

Fig. 1. Schematic representation of the proposed prediction model. If both the peptide-reactivity assay (e.g. DPRA) and ARE-dependent keratinocyte (KC) activation assays (LuSens or KeratinoSens™) yield negative results, then the substance is considered to be nonsensitizing. If substances are predicted to be sensitizing in the dendritic cell (DC) activation assay, mMUSST, the substance is then considered to be a sensitizer. If the predictions are conflicting, or if the h-CLAT is used instead of the mMUSST, a pragmatic 'weight of evidence' approach is used: Any two of the three tests rule the overall result (any two assays must be positive to rate the substance as a skin sensitizer and any two assays must be negative to rate the substance as a nonsensitizer).

need to develop test strategies comprising the most reliable methods in order to cover the biological complexity of the skin sensitization process. While the successful intralaboratory method validation of three *in vitro* methods reported earlier (Bauch et al., 2011) are presented here, a number of other methods for the prediction of skin sensitization were assessed but not further used due to practical feasibility, e.g. limitations such as dealing with human blood. The assays described in this study have been extensively studied, and most of them are transferable to other laboratories (the interlaboratory validation for LuSens is pending) and thus are considered to be reliable and workable test systems. The LuSens assay is similar to the KeratinoSens™ assay and, according to the data produced for the 54 test substances in this assay, it would be possible to use the KeratinoSens™ assay interchangeably without major differences in predictive capacity. Besides being used for the intralaboratory validation of the individual assays, the test results were also used to develop test strategies by combining different tests which cover key elements of the complex biological pathways leading to sensitization, which lead to a reliable prediction of skin sensitizers.

In the initial phase of this study, six different *in vitro*, *in chemico* and *in silico* assays were assessed for their performance to predict the sensitizing potential of test substances. Test substances were all in a molecular weight range of less than 400 g/mol for which skin penetration can be assumed. Hence dermal penetration was not assessed experimentally in this study. The 54 substances included in the strategy development were sufficiently soluble in vehicle and culture medium and did not interfere with the detection methods. The test methods used in this study address several events in the skin sensitization process: (1) the peptide reactivity of test substances, which was assessed using the *in chemico* DPRA, (2) ARE reporter gene based assays, namely the LuSens assay and KeratinoSens™ assay, for indirect protein reactivity and concomitant evaluation of keratinocyte responses due to the activation of Keap-1/Nrf2 signaling pathway and induction of genes under the control of ARE; and (3) dendritic cell activation; this was addressed using dendritic cell-like cell lines: the mMUSST assay and h-CLAT assays (Ashikaga et al., 2006; Bauch et al., 2011). The intralaboratory validation study was conducted with a set of 54 substances comprising 32 skin sensitizers with diverse potencies and 22 nonsensitizing industrial substances and cosmetic ingredients that were subjected to all six tests. This study was an extension of the study previously published and which assessed the individual methods (with the exception of OECD QSAR toolbox and LuSens assay) when testing the substances described by the performance standards proposed by ICCVAM and 2,4,6-trinitrobenzene sulfonic acid (Bauch et al., 2011; ICCVAM, 2009; OECD, 2010). These performance standards recommend chemicals to be used to allow a more rapid validation of modifications to the LLNA protocols. The assays and results are summarized as an overview in Table 5. The substances cover fourteen different structural alerts of the OECD QSAR toolbox for protein binding.

Each individual assay offered sensitivities between 64% and 89% or 56% and 83%, and specificities between 77% and 100% or 71% and 100% and the overall accuracies ranged from 76% to 86% or 70% to 81% when compared to human data or LLNA data, respectively. Among single assays, the DPRA showed the highest predictivity (human: 89% sensitivity, 82% specificity and 86% accuracy) and correctly predicted the sensitizing potential of 43 of 50 substances when compared to human data. This was somewhat higher than the sensitivity of 81%, specificity of 91% and accuracy of 84% reported for cysteine reactivity assay by Gerberick and co-workers in 2007 (Gerberick et al., 2007). In this study the DPRA identified all pro- and prehaptens correctly with the exception of aniline. These correct predictions were unexpected as the assay does not include metabolic activation, i.e. these results should actually be considered to be false positives if the mechanistic accuracy of the assay itself was to be assessed. For particularly sensitive substances, oxygen dissolved in the incubation medium may be sufficient to result in peptide reactivity. It is therefore speculated, that the oxygen in the sample mixture is sufficient to transform prehaptens to haptens although the transformation from prohaptens to haptens cannot be explained.

The OECD QSAR Toolbox Version 2.0 was used to predict the protein reactivity of the parental molecule structures. The predictions yielded no false positive results but incorrectly classified 10 substances as being non-skin sensitizers. Among the false negatives, eight pro- and prehaptens were not identified as sensitizing substances. If the metabolic pathway and/or correct metabolic activation of the substances is known, the accuracy of this *in silico* method can be increased. As data on metabolites of the pro- or prehaptens tested in this study is available, which is rarely the case, further analyses were performed using the OECD QSAR Toolbox Version 2.0. The skin metabolism module predicts metabolites and these can be again processed for their protein reactivity. Six of eight pro- or prehaptens were then correctly detected as having a skin sensitizing potential since at least one protein binding metabolite was predicted (data not shown). The skin metabolism module was then applied to the 54 substances evaluated in this study and protein binding metabolites were also occasionally predicted for nonsensitizing substances. Therefore, application of the skin metabolism module did not improve overall predictivity. As the metabolites for all substances are not known, the potential metabolites calculated by the program may not sufficiently reflect those found *in vivo*. Application of the protein binding module to assess the protein reactivity of the parent substance did offer sufficiently good predictivities to be used for initial assessments of the potential protein binding, although a better understanding and integration of the skin metabolism could improve the accuracies.

Each method has limitations regardless of whether animal, human or non-animal test and that usually defines its applicability domain. Probably the major limitation of the LuSens, KeratinoSens™, mMUSST and h-CLAT assay, and any submerse cell culture system without an air–liquid interface exposure, is the water solubility of the test substances. In general, the test substance must be soluble in the aqueous culture media to obtain reliable results, albeit limited amounts of a solubilizer such as 1% DMSO can be used. Any precipitation of the test substance can cause inaccurate results. Nukada and co-workers described that water-insoluble substances are the limitation of DC activation assays and decrease their sensitivity (Nukada et al., 2011). For this reason, three test substances were excluded from the original set of 59 test substances: Chlorobenzene, 1-bromobutane and geraniol. No appropriate alternative vehicle was identified that did not have a concomitant negative effect on the cell viability and the basal expression of the CD86 and/or CD54 cell surface marker. A further two substances (Tween 80 and Triton-X 100) were excluded as, in our hands, foam formation interfered with the flow cytometric analyses in spite of the cells being washed prior to analysis. Nickel has been reported to be a skin sensitizer in the h-CLAT but this seems to be dependent from which source the cells were obtained (Kosaka et al., 2008). This may also be one reason why some results generated in this study differed from those reported by Ashikaga et al. (2010). An additional limitation of cell based assays is the metabolic capacity. Cellular systems like dendritic cell-like cell lines are reported to lack some metabolic competence (Chipinda et al., 2011; Roggen et al., 2008). As prehaptens are not reactive towards proteins but need to undergo spontaneous chemical reactions to form a reactive hapten, and prohaptens require a metabolic transformation to form a protein-reactive hapten (Lepoittevin, 2006; Smith and Hotchkiss, 2001) these aspects are not

always sufficiently covered by the test system used. This is also one of the major disadvantages of the *in chemico* tests, e.g. the DPRA, although in this study most prohaptens were unexpectedly identified. Both prehaptens were predicted correctly probably due to auto-oxidation (in air). In the absence of metabolic activity (either intrinsic or added), prohaptens are typically identified as being non- or weak skin sensitizers. The DPRA as performed here uses peptide depletion as a measure of peptide reactivity but does not identify and measure the newly formed peptide adducts – the representatives of the ultimate antigens. Ball and co-workers reported that some surfactants lead to the dimerization of peptides thereby leading to peptide depletion without adduct formation (Ball et al., 2011). This was also the case for SDS which would explain the false positive result obtained in the DPRA for SDS in this study. These substances would then be incorrectly classified as being skin sensitizers. This has also been addressed by Natsch and co-workers, who then further developed the method by using a cor1 peptide which includes additional cysteine residues, followed by a LC–MS analysis. This modifications enable the identification of the parent peptide, and adducts and oxidization products thereof (Natsch and Gfeller, 2008).

In this study, the newly developed LuSens assay showed an activation of the reporter gene after exposure to all pro- or prehaptens except eugenol. The U937 cells (mMUSST assay) were able to respond to all pre-or prohaptenic substances with the exception of isoeugenol and cinnamic alcohol, whereas the THP-1 cells (h-CLAT assay) responded to all pro- and prehaptenic substances tested in this study. None of these assays specifically included addition of a metabolizing system, for example S9 mix. Since the prohaptens and prehaptens were correctly identified as skin sensitizers, this may indicate that endogenous metabolic capacity in the cell-based systems is sufficient and/or that the assays allow for adequate non-enzymatic oxidation. For example, the prehapten 4-phenylenediamine is known to be non-enzymatically oxidized to a Bandrowski's base. Several researches have reported initial endeavors to incorporate metabolic activation in both the cell based assays and DPRA. Gerberick et al. (2009) further developed the DPRA to incorporate a certain degree of metabolic activation via the addition of horse radish peroxidase. Chipinda and co-workers detected an activation of THP-1 cells by prohaptens such as benzo[*a*]pyren, carvoxime and cinnamic alcohol in the presence of a rat-liver S9 fraction (Chipinda et al., 2011). Schreiner and co-workers described a co-culture based cell system using primary keratinocytes and dendritic cells. With this system they were able to obtain positive responses with prohaptens (Schreiner et al., 2007, 2008). Additionally, integration of reconstructed skin models into test strategies could be used to provide metabolic activation (Jaeckh et al., 2011; Oesch et al., 2007; Pendlington et al., 2008). Skin models also offer the advantage of having a skin barrier function which would incorporate the factor of dermal penetration and bioavailability into the model and would also facilitate the application of poorly soluble test substances.

Despite the limitations, each individual assay has been shown to generate results with good predictivities for a large number of substances (Gerberick et al., 2007; Natsch and Emter, 2008; Python et al., 2007; Sakaguchi et al., 2007; Ashikaga et al., 2010). The goal of this study was therefore not primarily to assess individual predictivities but to select and combine assays according to rational models. In general, models which answer mechanism specific questions on the sensitization process should ideally be based on steps derived from the biological pathway, as this can be of use when dissecting the individual events leading to an adverse event, e.g. those used in the proposed prediction model. In order to have all options, a test battery of three tests, namely a peptide reactivity assay (e.g. DPRA or the LC–MS based peptide reactivity assay), an assay based on the activation of ARE related genes (e.g. the KeratinoSens™ or LuSens assays), and an assay based of antigen presenting cell activation (e.g. the mMUSST or h-CLAT assays) is proposed. Depending on the selected model, an optimized order of tests (a prediction model) can be designed to make testing more efficient. The model developed uses key steps in the sensitization pathway to assess the sensitization potential. For classification purposes without the use of animal testing, we propose using both the direct and indirect reactivity assays ("if both are negative") to identify nonsensitizers and the DC activation test mMUSST ("if positive") to determine the sensitization potential. If the predictions are contradictory, or if the h-CLAT is used instead of the mMUSST, then a weight of evidence approach ("2 out of 3") should be used. This model strives for a high overall accuracy of the prediction of a skin sensitizing potential of a substance in humans, which has been the aim of most *in vitro* assays developed so far. The accuracy (94%) of the prediction model is equivalent and even surpasses that of the LLNA, and high sensitivities (93%) and specificities (95%) were obtained in this study. This indicates an excellent approach to reducing animal testing for the endpoint sensitization.

The model cannot only be used for classification purposes but also for other applications, e.g. parts of the model can also be used to address concerns that the product developer may encounter, e.g. limited amount of substance available for testing, large number of substances need to be screened. During the screening process it is usually important to primarily identify possible skin sensitizers or to ensure that the probability of the substances being nonsensitizers is given. The model optimizes the predictivity to yield either a high sensitivity or a high specificity – obviously two questions of different nature and profoundly different consequences. A high sensitivity is used to reliably exclude a sensitizing potential, this would be addressed by the reactivity assays and/or activation of the Keap-1/Nrf2 signaling pathway (e.g., DPRA, LuSens). A high specificity reduces the number of false positive results, which can be of importance when making decisions during product development when an otherwise promising candidate is prematurely rejected; this would be addressed by the dendritic cell activation assay (mMUSST).

The models presented here are not yet able to classify substances into weak, moderate, strong or extreme skin sensitizer; i.e. do not allow assessments of potency. Potency is, however, an important parameter used in risk and safety assessments. The LLNA still remains the gold standard for potency assessments. Potency is predicted via estimated concentration leading to a specified fold induction of the proliferative responses of the lymph node cells. Kimber and coworkers described thresholds in the LLNA to categorize skin sensitizer as weak, moderate, strong and extreme skin sensitizer (Kimber et al., 2003). For classification according to the global harmonized system (GHS), it is of interest to distinguish strong and less strong skin sensitizers (Category 1A or 1B). For the *in chemico* method DPRA, Gerberick and coworkers drafted a decision tree model to classify the reactivity of substances according their mean peptide depletion ability into minimal, low, moderate, or high (Gerberick et al., 2007). Jowsey and co-workers published a hypothetical prediction model where dose–response and a relative activity in test systems are assigned a score for to be used in classification of skin sensitizers. This proposal needs to be further corroborated using experimental data (Jowsey et al., 2006). Natsch and coworkers enhanced predictivities by using a combination of an ARE-reporter assay, peptide reactivity assay, and an *in silico* model (TIMESS Natsch et al. (2009)). A recently published integrated testing strategy based on experimental data (Jaworska et al., 2011), proposes a way of integrating different *in silico*, *in chemico* and *in vitro* methods that leads to generation of networks. What remains to be seen is whether a suitable predictive model can be developed to use the combination of methods used in this study to allow potency to be assessed.

5. Conclusions and outlook

To the best of our knowledge, this study is the first to test the same 54 substances in all six methods presented in this study and to develop test strategies suitable for the reliable prediction of sensitizing potential for a substantial number of substances. The test battery reflects key steps in the sensitization process namely protein reactivity, Nrf2/ARE activation as well as dendritic cell activation. The accuracy of the prediction model proposed exceeded that of the LLNA for set of substances tested. These very promising results point to a possible future replacement of animal testing while maintaining the same degree of accuracy and predictivity for human skin sensitizers. Future developments will need to focus on applicability domains and potency. Whether the development of a prediction model to assess the potency of skin sensitizer using the tests described in this study is feasible will be a challenge.

6. Conflict of interest statement

Other than employment, the authors are not aware of any conflicts of interest.

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Corrigendum

Corrigendum to "Putting the parts together: Combining in vitro methods to test for skin sensitizing potentials" [Regul. Toxicol. Pharmacol. 63 (2012) 489–504]

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The authors regret the following information should have been included in the original manuscript:

Since the predictivity of the combination of assays is directly linked to the performance of single assays, the following amendments need to be included:

In Table 2, the EC1.5 for nickel chloride in the LuSens assay should read 'below threshold" and the '+' (positive) in Table 5 should read "–" (negative). As a consequence, there is a slight change in the predictivities: the LuSens predictivities compared to human data should read: 86%, 77%, 83%, 81% and 82%, and compared to the LLNA: 81%, 76%, 88%, 65% and 79%; the combination of the LuSens with the h-CLAT compared to human data should read: 93%, 68%, 79%, 88% and 82%, and compared to the LLNA: 86%, 65%, 84%, 69% and 79% for sensitivity, specificity, positive prediction value, negative prediction value and accuracy, respectively. The predictivities of other combinations of LuSens were not affected.

The authors would like to apologise for any inconvenience caused.

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7 HAPTENIZATION *IN VITRO*

7.1 CHARACTERIZATION OF HAPTENIZATION OF CHEMICAL SENSITIZER ON THE CELL SURFACE

Bauch C.; Dearman R.J.; Kimber I., van Ravenzwaay B.; Landsiedel, R.: *Characterization of haptenization of chemical sensitizer on the cell surface*

Submitted at Toxicology In Vitro; Submitted on the 03rd of February: (Submission letter see Appendix)

The manuscript "Characterization of haptenization of chemical sensitizer on the cell surface" has been submitted and is currently under review. It describes the investigation and characterization of the reaction of chemical sensitizers with proteins on the cell surface of two DC like cell lines, U-937 and THP-1, both used in the previously described DC activation assays. The model sensitizer 1-chloro-2,4-dinitrobenzene (DNCB) was used to investigate whether the treatment of cells leads to a) an interference with antibody binding indicated by reduced level of bound antibody or b) removal of conjugated protein from the cell surface by internalization and degradation of the conjugated protein. The initial hypothesis was that reaction of chemicals with proteins leads to inevitable changes of the antibody binding site and thus to a loss of antibody affinity. Three possible interactions with antibody binding can be described (Figure 5): covalent binding of the chemical may block antibody recognition (1) directly by changing polarity of hydrophobicity of the epitope, (2) indirectly by conformational changes of the entire epitop or (3) sterical hindrance of antibody binding site.

The selection of the cell surface determinant was accord to the following criteria: 1) either constitutive expression of the protein on the cell surface or possibility to control expression levels and 2) availability of antibodies for flow cytometric analyses. Both cell lines were reported to express CD14 and CD54 constitutively or upon stimulation. Although CD54 is usually quantified to determine activated DC, in this investigation both markers were used as model protein to assess reactivity of chemicals with cell surface proteins.

Experiments with DNCB indicated that a 2 h incubation had a dose dependent effect on detectable levels of both markers. Irreversible metabolic inhibition of cells with sodium azide or fixation with formaldehyde abrogated these effects, whereas reversible inhibition of endocytosis with on ice treatment shifted the dose response towards higher concentrations. Triggering protein turnover with specific antibodies, so called antibody modulation, indicated protein uptake under standard conditions at RT but inhibited uptake for on ice and for sodium azide treated cells. These findings, especially the controversial findings for on ice treated cells, suggest that reduced levels of detected proteins are likely to be a combination of protein uptake and reduced antibody binding. In addition it is likely that the process of protein conjugation on the cell surface and the internalization of the altered protein is involved in the process of dendritic cell activation.

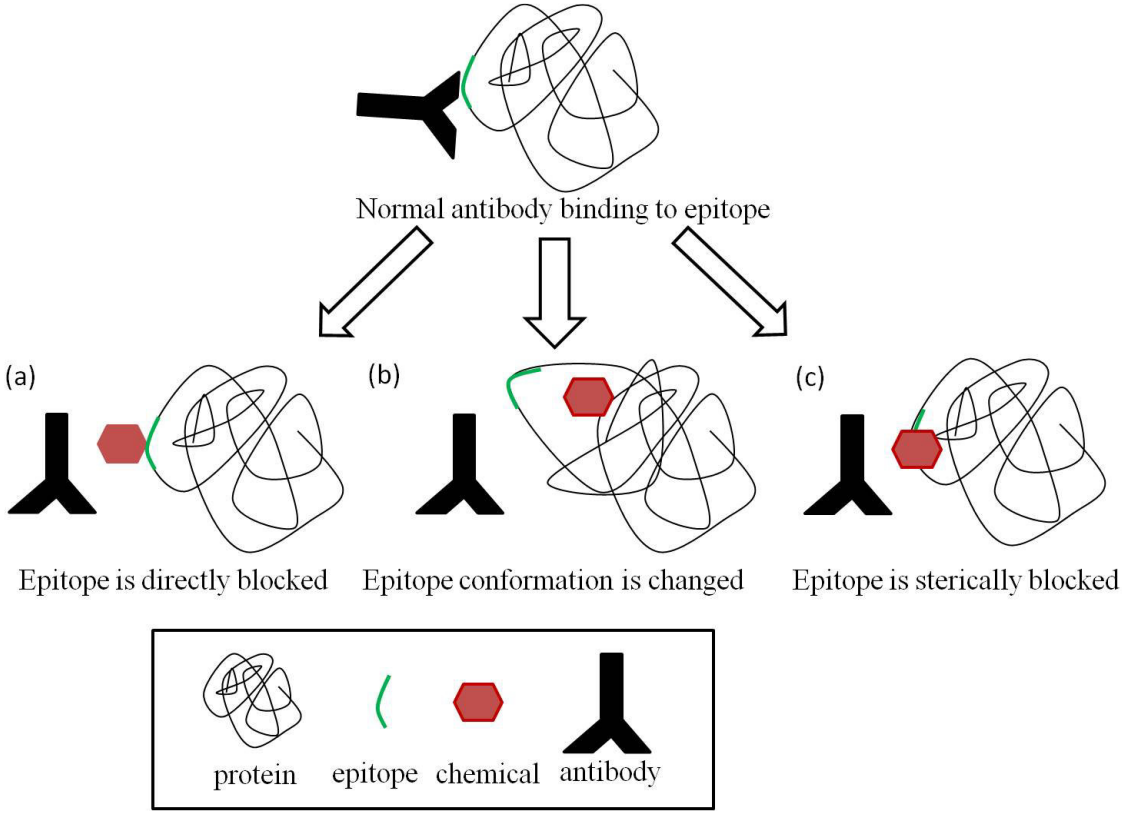

Figure 5: Hypothesis of chemical treatment, blocked antibody recognition and antibody binding.
Covalent binding of the chemical may (a) change the epitope directly and thus blocks recognition; (b) cause conformational changes and alters the epitope or (c) sterically block the antibody binding site.

It was further investigated whether these effects on cell surface proteins are restricted to skin sensitizers and the associated protein conjugation or if the presence of any chemical may trigger changes in and uptake of proteins. Therefore the sensitizers *para*-phenylenediamine, oxazolone, *para*-benzoquinone and formaldehyde were tested. The known irritants salicylic acid and SDS were selected as additional substances in order to show that the protein reactivity of sensitizers triggers changes in surface protein level but not the presence of a chemical. Increasing concentrations of *para*-phenylenediamine led to a corresponding decrease of the assessed cell surface determinant. The tests with oxazolone, *para*-benzoquinone and formaldehyde showed a decrease of the assessed protein expression when applied in lower concentrations and an increase at higher concentrations. For the latter effect was rationalized with the up-regulation of the chosen cell surface determinant by activation of DC It was assumed that the detectable reaction of chemicals with the protein depends an equilibrium of newly synthesized protein and conjugation reaction. As expected, treatment with irritants did not affect the protein detection at subtoxic concentrations. Reduced cell viability especially after treatment with SDS lead to a marked reduction of the detectable protein not only due to reduced cell number but also being able as detergent to extract protein from the cell membrane.

These results indicate that, although further validation is required, an antibody binding test may be used for assessment of chemical sensitizers and their effect on cell surface proteins. However, further work is still

needed. For instance the protein determinant could be exchanged for a surface protein that is less responsive to DC activation. This may reduce variability of results caused by protein expression due to activation of cells. In addition, cell activation could be avoided by investigating cell lines other than DC, that are less responsive to sensitizing chemicals.

The experimental work as well as the data evaluation of this project was solely performed in context of this PhD project. None of the experiments were performed by others.

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Abstract: The ability of chemicals to interact with host proteins is a pivotal step in the acquisition of skin sensitization. For this reason evaluation of the electrophilic properties of chemicals in silico, or of protein/peptide binding activity in vitro, have attracted interest as bases for the development of novel approaches to hazard identification. Described here is an alternative strategy wherein we have measured the association of chemicals with proteins expressed on the plasma membrane of cells. We have shown that sensitizing chemicals such as 2,4-dinitrochlorobenzene (DNCB) and p-phenylenediamine reduce recognition by antibody of specific cell surface determinants expressed by THP-1 and U-937 cells. In contrast, non-sensitizing chemicals, such as sodium dodecyl sulfate, were without effect.
In addition, it was shown that irreversible inhibition of cell viability, or of metabolic activity, reduced or prevented the inhibition of antibody reactivity with allergen-treated cells. Intracellular detection of dinitrophenol, the leaving group of DNCB, suggested that interaction of chemical allergens with membrane proteins inhibits antibody detection through both increased internalization of target molecules, as well by obscuring or modifying epitopes. Although for other contact allergens the results were variable and less persuasive, the approach shows some merit and other membrane markers may prove more tractable.

Characterization of haptenization of cell surface proteins by skin sensitizing chemicals

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Abstract

The ability of chemicals to interact with host proteins is a pivotal step in the acquisition of skin sensitization. For this reason evaluation of the electrophilic properties of chemicals *in silico,* or of protein/peptide binding activity *in vitro,* have attracted interest as bases for the development of novel approaches to hazard identification. Described here is an alternative strategy wherein we have measured the association of chemicals with proteins expressed on the plasma membrane of cells. We have shown that sensitizing chemicals such as 2,4-dinitrochlorobenzene (DNCB) and p-phenylenediamine reduce recognition by antibody of specific cell surface determinants expressed by THP-1 and U-937 cells. In contrast, non-sensitizing chemicals, such as sodium dodecyl sulfate, were without effect.

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Abbreviations

ACD – allergic contact dermatitis; DMSO – dimethylsulfoxide; DNCB – 1-chloro-2,4-dinitrobenzene; DNP – dinitrophenol; DPRA – Direct Peptide Reactivity Assay; FCS – foetal calf serum; FITC – fluorescein isothiocyanate; LLNA – local lymph node assay; MFI – mean fluorescence intensity; PBQ – para-benzoquinone; PBS – phosphate buffered saline; PI – propidium iodide; PMA - phorbol-12-myristat-13-acetate; PPD – para-phenylenediamine; RT – room temperature; SDS – sodium dodecyl sulfate

Introduction

There is a continuing interest in the development of alternative (non-animal) methods for the safety testing of chemicals, drugs and other products (European Commisson 1986; Goldberg & Hartung 2006). Although for many years tests such as the guinea pig maximization test (Magnusson & Kligman 1969), and the murine local lymph node assay (LLNA) (Basketter et al. 1996; Kimber & Basketter 1992) have been used successfully for the identification of skin sensitizing chemicals, the focus of attention has turned now to the design of approaches that obviate the need for animals. The development of reliable and reproducible *in vitro* or *in silico* methods for skin sensitization hazard identification and characterization is extremely challenging; not least because of the need to model accurately the cellular and molecular processes that together orchestrate the acquisition of sensitization. Indeed, it may prove that no single test may be able to independently replace the proven animal assays (Roggen et al. 2008; Ryan et al. 2001). Despite these challenges progress has been, and is being, made (dos Santos *et al.* 2009; Mehling *et al.* 2012). Among the approaches that appear to show the greatest promise are those based on the activation/maturation of dendritic cells (DC) or DC-like cell lines *in vitro,* the activation by chemicals of discrete signaling pathways, or measurement of the ability of test chemicals to associate with peptides (peptide binding assays) (Ashikaga et al. 2006; Nukada et al. 2012) For the acquisition of skin sensitization chemicals must be able to form stable immunogenic associations with host peptides or proteins. It is, therefore, axiomatic that contact allergens must be naturally electrophilic, or should be converted to an electrophilic species in the skin (Ahlfors et al. 2003; Dupuis & Benezra 1982; Jaeckh et al. 2012; Lepoittevin et al. 1998). It is for this reason that *in vitro* assays, such as the Direct Peptide Reactivity Assay (DPRA) (Gerberick *et al.* 2004; Gerberick *et al.* 2007), have attracted considerable interest. In parallel there is interest also in the development of Structure-Activity methods in which the aim is to predict the electrophilic potential of chemicals from a consideration of structural characteristics (Aptula & Roberts 2006; Patlewicz et al. 2008; Roberts et al. 2007).

In 2009 Hirota and co-workers demonstrated that exposure of the human acute monocytic leukemia DC-like cell line THP-1 to the skin sensitizer 1-chloro-2,4-dinitrobenzene

(DNCB) resulted in a reduction in free cysteine and lysine residues on the cell surface and activation via MAP kinases (Hirota *et al.* 2009). In addition, treatment with DNCB-bovine serum albumin conjugates failed to impact on the level of free cysteine and lysine residues on the cell surface and did not activate THP-1 cells (Hirota *et al.* 2009). Based on those observations it is reasonable to assume that electrophilic chemical allergens will have the ability to interact not only with soluble proteins, but also with proteins expressed by cells at the plasma membrane. The implication is that assessment of the interaction of chemicals with cell-associated proteins might provide the basis of an alternative assay for the identification of contact allergens. Certainly it has been shown that cell surface proteins can be targets for haptenization (Hirota *et al.* 2009; Hopkins *et al.* 2005), and that this in turn may contribute to acquisition of sensitization. Irrespective of the contribution that haptenated plasma membrane proteins may make to the initiation of skin immune responses (Megherbi *et al.* 2009), we speculated that it may be possible to detect interactions of contact allergens such as DNCB with cell surface proteins as a function of changes in antibody recognition of altered membrane-bound "reporter" proteins using flow cytometry. The binding of an antibody to a protein on the cell-surface may be blocked directly due to masking of the epitope, by association of the chemical with protein or as the result of conformational changes. Alternatively, it is possible that the exposure of the cell to a chemical could change the cellular expression of proteins.

Against this background, the aim of these investigations was to determine whether selected sensitizing and non-sensitizing chemicals are able to influence the recognition of discrete plasma membrane proteins by specific antibodies and to consider how such effects are mediated. For this purpose the human DC-like cell lines U-937 and THP-1 have been used. These studies form part of a broader experimental strategy to identify and evaluate novel approaches to the identification and characterization of skin sensitizing chemicals and to explore how such assays may be used in concert to provide new paradigms for safety assessment.

Materials and Methods

Cell line maintenance and culture

The human histiocytic lymphoma cell line U-937 and the human acute monocytic leukemia cell line THP-1 were maintained in suspension culture in RPMI 1640 medium (Invitrogen Inc., Carlsbad, CA) supplemented with 10% foetal calf serum (FCS; PAA, Linz, Austria), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen Inc.) and 2 mM L-glutamine (Sigma/Aldrich, Gillingham, UK) (cell culture medium). Cells were subcultured twice a week as follows. Cells were pelleted by centrifugation at 1000 rpm, 5 min at room temperature (RT). The cell pellet was resuspended in 5 to 10 mL fresh cell culture medium and counted using a haemocytometer and trypan blue (0.4% solution, Sigma/Aldrich) for exclusion of dead cells. For U-937 cells, cell density was adjusted to $0.25x10^6$ cells per flask for 4 days of culture or $0.5x10^6$ cells per flask for 3 days of culture; both in 20 mL cell culture medium. Whereas for THP-1 cells, the initial cell density was $3x10^6$ cells per flask for 4 days of culture or $4x10^6$ cells per flask for 3 days of culture; both in 20 mL cell culture medium.

Activation of cells with phorbol-12-myristat-13-acetate (PMA)

Cells were harvested by centrifugation (1000 rpm, 5 min, RT) and resuspended in 5 to 10 mL fresh cell culture medium. Cell number was assessed using a haemocytometer and trypan blue exclusion, adjusted to $0.33x10^6$ cells/mL and aliquotted into 24 well tissue culture plates with 0.9 mL cell suspension per well. PMA (Sigma/Aldrich) was stored as stock solution (10 mg/mL in DMSO) and was further diluted down to 0.01 µg/mL PMA in medium for U-937 cells or 0.1 µg/mL PMA in medium for THP-1 cells. Cells were treated with 0.1 mL medium alone (resting cells) or with an equal volume of PMA solution followed by 72 h (U-937 cells) or 24 h (THP-1 cells) incubation under standard cell culture conditions. After being activated cells were harvested by centrifugation (1200 rpm, 5 min, RT) and resuspended in 0.5 mL fresh medium without FCS and aliquotted into a U-bottomed 96 well tissue culture plate for treatment with chemical or for antibody staining for flow cytometric analyses.

Inhibition of metabolic activity

A number of methods were used to inhibit metabolic activity. Cells were fixed by incubation with 4% formaldehyde solution (7.6 mL water, 1.4 mL phosphate buffered saline [PBS; Fisher scientific, Waltham, MA], 1 mL 37% formaldehyde solution [CAS #: 50-00-0; 37%; Sigma/Aldrich]) for 10 min on ice. Alternatively, cells were incubated on ice for at least 60 min (ice cold reagents were used) in the presence or absence of 20 mM sodium azide (VWR BDH prolabo, Radnor, Pennsylvania). Cells were washed at least once with either PBS or serum free medium by centrifugation (1200 rpm, 5 min, 4°C).

Chemical treatment of cells

Cells were harvested, counted and pre-treated on ice or with 20 mM sodium azide as described above. Control cells for RT treatment were maintained in the 37°C incubator during inhibition steps. After pre-treatment (see above) cells were aliquotted into U-bottomed 96 well plates and pelleted by centrifugation at 1200 rpm, 5 min at 4°C. Supernatants were discarded. Chemicals (DNCB [CAS #: 97-00-7; ≥98%;], para-phenylenediamine [PPD; CAS #: 106-50-3; ≥99%;], para-benzoquinone [PBQ; CAS #: 106-51-4; ≥99.5%;], 3-ethoxymethylene-2-phenyl-2-oxazoline-5-one [oxazolone; CAS #: 15646-46-5; ≥90%;], formaldehyde, salicylic acid [CAS #: 69-72-7; ≥99%;], sodium dodecyl sulfate [SDS; CAS #: 51-21-3; ≥98.5%;]) were pre-tested for solubility in either medium or DMSO. All chemicals were supplied by Sigma/Aldrich. Stock solutions were prepared in either medium or sterile DMSO. The top concentrations of chemicals utilized were dependent on maximum solubility in medium and/or DMSO. Stock solutions in DMSO were further diluted 1:200 in serum free medium for a final DMSO concentration of 0.5%. In parallel, cells were treated with medium or 0.5% DMSO vehicle alone as controls. The various chemicals at a range of concentrations were added (100 µL per well) and cells were incubated for 2 h and/or 24 h at RT. Following the incubation with chemicals, cells were washed three times with cold 5% FCS in PBS (wash buffer) and processed for staining against cell surface proteins or dinitrophenol (DNP) molecules.

Flow cytometric analyses of cell surface markers

Antibodies (anti human CD86 [clone FUN-1; mouse IgG1 κ; BD Pharmingen, San Diego, CA]; anti human CD54 [clone HA58; mouse IgG1 κ; BD Pharmingen]; mouse IgG1 κ isotype [clone MOPC-21; mouse IgG1 κ; BD Pharmingen]; anti human CD14 [clone 61D3, mouse IgG1 κ; ebioscience, San Diego, CA]) were diluted in wash buffer to a final concentration of 10 µg/mL. For cell surface staining, 50 µL of antibody solution was added to the appropriate well followed by 30 min incubation on ice. After incubation cells were washed three times with wash buffer by centrifugation (1200 rpm, 5 min, 4°C). Secondary fluorescein isothiocyanate (FITC) labelled antibody (polyclonal goat anti-mouse IgG; Dako, Glostrup, Denmark) was diluted in wash buffer to a concentration of 4 µg/mL. Cells were stained for 30 min on ice in the dark followed by three washing steps with wash buffer by centrifugation (1200 rpm, 5 min, 4°C). After the final washing step cells were resuspended in running buffer (1% FCS/0.05% sodium azide/PBS) and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuestPro software (Becton Dickinson). Cell viability was assessed in parallel with cell surface marker detection by staining the nuclei of dead cells using 1 µg/mL propidium iodide (PI; Sigma/Aldrich) prior to flow cytometric analyses.

Flow cytometric analyses of DNP expression

For staining of DNP molecules on the cell surface, rabbit anti-DNP antibody (Invitrogen Inc.) and rabbit IgG isotype control (R&D systems, Minneapolis, MN) were diluted in wash buffer to a final concentration of 1 µg/mL. Cells were stained for 30 min on ice followed by three washing steps with wash buffer by centrifugation (1200 rpm, 5 min, 4°C). Staining with 10 µg/mL secondary goat anti-rabbit IgG conjugated with AlexaFluor® 488 (Invitrogen Inc.) was performed on ice and in the dark for 30 min followed by three washing steps with wash buffer by centrifugation (1200 rpm, 5 min, 4°C). After the final washing step, cells were resuspended in running buffer and analyzed using a FACSCalibur flow cytometer and CellQuestPro software. Dead cells were

excluded using PI staining as described above. For intracellular staining for DNP, rabbit anti-DNP antibody and rabbit IgG isotype control were diluted to a final concentration of 1 µg/mL in 0.3% saponin (from Quillaja Bark, Sigma/Aldrich) containing wash buffer. Cells were stained for 30 min on ice followed by three washing steps with 0.1% saponin containing wash buffer by centrifugation (1200 rpm, 5 min, 4°C). Staining with 10 µg/mL secondary goat anti-rabbit IgG conjugated with AlexaFluor® 488 in 0.3% saponin/wash buffer was performed on ice and in the dark for 30 min followed by three washing steps with 0.1% saponin/wash buffer by centrifugation (1200 rpm, 5 min, 4°C). After the final washing step, cells were resuspended in running buffer and analyzed using a FACSCalibur flow cytometer and CellQuestPro software.

Antibody-induced modulation of cell surface proteins

Cells were harvested, counted and pre-treated on ice or with 20 mM sodium azide as described above. Control cells (treated at RT) were maintained in the incubator. Anti-CD54 antibody was diluted in medium to a concentration of 10 µg/mL and added to the cells (50 µL/well). Cells were incubated for 0.5, 1, 1.5 and 2 h either on ice or at RT and then washed three times by centrifugation and stained with secondary FITC labelled goat anti-mouse IgG antibody for 30 min on ice in the dark followed by three washing steps with wash buffer. Flow cytometric analysis was performed using a FACSCalibur flow cytometer and the CellQuestPro software. Dead cells were excluded by PI staining as described above.

Data analyses

Flow cytometric data were analyzed with FlowJo® 7.6.1 software (TreeStar Inc., Ashland, OR).

Cell marker expression data are displayed with respect to chemical-induced changes in mean fluorescence intensity (ΔMFI) relative to medium control treated cells using following equation:

ΔMFI = (MFI antibody stained control cells minus MFI isotype stained control cells)

minus (MFI antibody stained test substance treated cells minus MFI isotype stained test substance treated cells)

Intracellular DNP content is displayed as the fold change in anti-DNP antibody binding (MFI values), relative to the background levels recorded in medium control treated cells in the absence of DNCB treatment (defined as 1). This parameter was chosen (rather than Δ MFI) due to the relatively low level binding recorded in control cells in the absence of DNCB.

Antibody modulation is displayed with respect to CD54 expression after 0.5 h antibody staining on ice and antibody-induced changes in MFI were calculated using following equation:

ΔMFI = (MFI antibody stained 0.5 h on ice) minus

(MFI antibody stained at different time points and different conditions)

Unpaired Students' *t*-test was used for statistical analyses.

Results

Membrane marker profiles of U-937 and THP-1 cells

Initial experiments were conducted to characterize U-937 (Fig.1 panel a) and THP-1 (Fig. 1 panel b) cells with respect to cell surface protein expression on resting cells (i, ii, iii) and on cells after activation with PMA (iv, v, vi). Cells were stained for the monocyte marker CD14, the adhesion molecule CD54 and the co-stimulatory molecule CD86 and analyzed by flow cytometry. Resting U-937 cells (Fig. 1 panel a) did not express detectable CD86, whereas approximately 29% of cells expressed relatively low levels (mean fluorescence intensity; MFI = 3) of CD14 and the majority of cells (99%) expressed CD54 (MFI = 30). It was observed that expression levels with respect to MFI, particularly CD54, varied with passage number and time of culture period (data not shown). THP-1 cells (Fig. 1 panel b) failed to express constitutively CD86 or CD54. Approximately 65% of cells were CD14 positive (MFI = 18). Cells were cultured with PMA for 72 h (U-937; Fig. 1 panel a) and 24 h (THP-1; Fig. 1 panel b) and the impact on membrane marker expression examined. Incubation of U-937 cells with 0.01 µg/mL PMA induced CD86 (MFI = 10; 55% of cells) and upregulated CD54 (MFI = 123) expression whereas CD14 was down-regulated with respect to both MFI (7) and the frequency of positive cells (10%). Activation of THP-1 cells with 0.1 µg/mL PMA had little effect on CD86 (<1% positive cells), whereas expression of CD54 was markedly increased (MFI = 20; 66% of cells positive) and for CD14 a modest upregulation was detected (MFI = 28; 84% of cells positive). Given the pattern of membrane marker expression described above, further studies focused primarily on changes in expression of CD54 on both resting and PMA-activated cells

Influence of DNCB on membrane marker expression

In subsequent experiments, the impact of DNCB on expression of selected membrane determinants was examined. Cells were incubated with DNCB at RT, in order to parallel the conditions used in the DPRA and binding to both viable and formaldehyde fixed cells was investigated. The latter was explored as fixed cells would provide for more flexibility with respect to exposure conditions such as use of non physiological pH or solvents.

Resting and PMA-activated U-937 cells, and PMA-stimulated THP-1 cells (Fig. 1 panel c), were exposed to 5 mM DNCB formulated in DMSO for 2 h and CD54 expression profiles were examined by flow cytometry. PMA-activated THP-1 cells and U-937 cells (regardless of prior activation with PMA) displayed detectable levels of CD54 which were unaffected by culture with the vehicle control DMSO, whereas treatment with DNCB in each case caused the MFI histogram to shift to the left markedly, indicating down-regulation of CD54 expression. The ability of DNCB to alter expression or the detection of membrane bound CD54 was investigated further in dose response analyses (Fig. 2). In these experiments, cells were cultured for 2 or 24 h at RT in the presence of 0.05, 0.5, 5 and 50 mM DNCB, or DMSO vehicle alone, and cell viability and the extent of CD54 expression measured using flow cytometry. To facilitate direct comparisons between resting and PMA-activated cells and U-937 and THP-1 cells, CD54 levels are displayed as the change (Δ) in levels of expression of this molecule relative to those recorded on medium control-treated cells. The viability of unstimulated and PMA-activated U-937 medium control and DMSO treated cells remained at approximately 85% after culture for 2 h. The presence of DNCB caused a small, dose-related decrease in cell viability for both resting and PMA-activated U-937 cells, reaching approximately 73% at the maximum dose (50 mM DNCB), although such did not reach statistical significance (except 50 mM on PMA activated U-937 cells). On resting U-937 cells the presence of DMSO had little effect on ΔMFI of CD54, whereas treatment with DNCB resulted in a dose dependent decrease that reached statistical significance at 5 mM DNCB. As observed previously (Fig. 1), PMA-activated U-937 expressed higher levels of CD54 compared to unstimulated U-937 cells, and DNCB treatment resulted in a similar pattern of down-regulation of this molecule, although the maximal effect was more marked and displayed greater inter-experimental variability. Thus, after 2 h culture with 5 mM DNCB, significant decreases in CD54 expression were observed for both unstimulated and PMA-activated U-937 cells, with ΔMFI values of 40 and 88 recorded, respectively.

In parallel, the effect of DNCB on CD54 expression by THP-1 cells was investigated (Fig. 2). As resting THP-1 cells did not express detectable levels of this membrane marker (Fig. 1), PMA-activated cells only were examined for the effect of DNCB treatment on CD54

detection (Fig. 2). Despite activation with PMA, viability of control (medium-treated) and DMSO treated cells approached 90%. The presence of DNCB resulted in significant cell toxicity at doses of 5 and 50 mM (viability 80.5% ± 1.5% and 54.4% ± 9.0%; $p<0.05$). The extent of DNCB-induced changes in CD54 expression was considerably less marked than that observed for U-937 cells (maximal ΔMFI of ~5 versus maximal ΔMFI of ~88). However, a similar dose dependent reduction in CD54 expression was observed, even at concentrations that were without effect on cell viability (0.5 mM DNCB), although changes were not statistically significant.

Experiments were also conducted in which cells were incubated for 24 h with DMSO vehicle or with various concentrations of DNCB. DMSO itself did not reduce cell viability after 24 h, however, all concentrations of DNCB resulted in very marked cytotoxicity (<50% viable) for both unstimulated and PMA-activated U-937 cells and PMA-activated THP-1 cells (Fig. 2 ai and ii). DNCB treatment resulted also in a marked decrease in membrane expression of CD54, however, due to the extensive cytotoxicity such were not considered further (data not shown).

The impact of cell fixation on the ability of DNCB to modulate CD54 expression was also examined (Fig. 2). Resting and PMA-activated U-937 and PMA-activated THP-1 cells were fixed with 4% formaldehyde for 10 min prior to incubation with various concentrations of DNCB. Treatment with formaldehyde did not markedly affect baseline CD54 expression on either cell type. Baseline values were 104 ± 26 and 126 ± 40 for viable and fixed cells, respectively, whereas MFI values of 10 ± 4 and 8 ± 1 for viable and fixed PMA-activated THP-1 cells, respectively, were recorded. However, fixation completely abrogated the effects of DNCB on the detection of CD54 across the whole concentration range of chemical in both cell types (Fig. 2).

As the alternative cell surface marker CD14 was expressed constitutively by both U-937 and THP-1 cells without the requirement for PMA activation, the impact of DNCB treatment on levels of this membrane determinant was investigated also (Fig. 2 panel b). Data are displayed with respect to ΔMFI. Consistent with the effects on CD54, 2 h

incubation with DNCB resulted in a dose dependent decrease CD14 expression on both cell types, although with somewhat higher levels of inter-experimental variation and thus changes were not statistically significant.

DNCB-induced changes in CD54 expression: requirement for metabolic activity

These initial data served to demonstrate that interaction of cell membrane determinants with the reference skin sensitizer DNCB did indeed affect the detection of membrane markers monitored by flow cytometry and the binding of fluorescent-labelled antibodies and was relevant for several different cell types and proteins (CD54 and CD14). Subsequent experiments focused on resting U-937 cells and the CD54 molecule, as this combination resulted in the most robust changes, and addressed potential mechanisms for the allergen-induced loss of detectable CD54. Given that the influence of formaldehyde fixation on DNCB-induced changes in CD54 expression suggested that the effect required metabolically active cells, the impact of incubation temperature (on ice versus RT) and the effect of the metabolic inhibitor sodium azide were examined (Fig. 3). As observed previously (cf Fig. 2), treatment of U-937 cells with DNCB for 2 h at RT resulted in a dose-dependent decrease in detectable cell-surface CD54. Treatment on ice shifted the dose response curve to DNCB, such that the two lower doses were no longer effective and the maximal effect at the higher doses was also attenuated. Furthermore, prior treatment of the cells with sodium azide completely abrogated the effect of DNCB on CD54 expression.

The influence of these conditions on uptake of DNCB was also assessed. Reaction of DNCB with proteins results in the formation of DNP-labelled proteins which can be detected using a commercially available anti-DNP antibody. The antibody was not sensitive enough to detect cell surface DNP, but following saponization of cells it was possible to detect intracellular DNP staining following 2 h incubation of U-937 cells at RT with DNCB. There was some background staining (MFI of ~20-30, not shown) in the presence of medium or DMSO alone. A dramatic and dose dependent increase in anti-DNP antibody binding was observed following DNCB treatment at RT (approximately

10fold increase in binding compared with medium-treated control or DMSO-treated cells). The detection of intracellular DNP was abrogated by treatment on ice but was unaffected by treatment with sodium azide,

One possible mechanism of the DNCB-induced loss of CD54 expression is that it is due to turnover and internalization of the membrane molecule, stimulated by the binding of chemical to the protein. A well-known example of membrane protein internalization is that driven by binding of antibody and subsequent cross linking which results in uptake of the protein (Lane *et al.* 1991), designated as antibody-driven "modulation", although this phenomenon is usually recorded at physiological temperature (37^{o}C). In order to determine if the effects of DNCB on CD54 expression were likely to be due to a similar mechanism as that of modulation, the impact of sodium azide and temperature on antibody-driven modulation was examined. Therefore U-937 cells were incubated for up to 2 h with anti-human CD54 antibody on ice or at RT in the presence or absence of sodium azide (Fig. 4). In these experiments, for the purposes of calculating ΔMFI for all treatment conditions, control cells were incubated with antibody for 0.5 h on ice (defined as 0 ΔMFI), as this regimen is identical to the standard staining protocol used for the analysis of cell surface marker expression in this work. Incubation with anti-CD54 antibody at RT resulted in a marked time dependent modulation of CD54 expression, with significant loss of CD54 expression at each time point examined and a maximum ΔMFI value of 74 achieved after 2 h. Modulation was markedly attenuated in the presence of sodium azide, with only the longest incubation time (2 h) resulting in significant reduction in CD54 expression at some 50% of the level observed in the absence of sodium azide. Incubation on ice completely abrogated the ability of the antibody to modulate expression of CD54.

Changes in CD54 expression: impact of other skin sensitizers and irritants

In subsequent experiments the impact of treatment with additional chemical sensitizers and non sensitizers on the expression of the cell surface determinant CD54 on viable U-937 cells was investigated. The chemical sensitizers were selected on basis of having a

range of reaction mechanisms and strong to extreme potency according to LLNA classification (Kimber *et al.* 2003; Patlewicz *et al.* 2008). It has been reported that DNCB reacts with proteins via a nucleophilic substitution, whereas PPD and PBQ act as (Pro)Michael acceptors, oxazolone is able to undergo an nucleophilic acyl substitution whereas formaldehyde forms a so-called Schiff's base. Concentrations were chosen according the maximal soluble concentration in either medium or DMSO. Top doses used were as following: DNCB 50 mM, PPD 28 mM, oxazolone 4 mM, PBQ 9 mM, formaldehyde 33 mM, salicylic acid 7 mM and SDS 3 mM. Chemicals were diluted 1:10 (DNCB and irritants) or 1:3.33 and further 1:10 (skin sensitizers excluding DNCB) in medium or DMSO, respectively. Viable U-937 cells were treated with increasing concentrations of the test substances for 2 h at RT and analyzed for changes in CD54 expression (Fig. 5). Treatment with PPD (concentration range of 0.01 – 28 mM) resulted in a dose dependent decrease in CD54 at concentrations higher than 0.09 mM, although no significant effects on cell viability were detected. For oxazolone (0.001 and 4 mM), PBQ (0.003 and 9 mM) and formaldehyde (0.01 and 33 mM), dose dependent decreases in CD54 expression were recorded in the lower ranges of concentrations tested whereas exposure of cells to higher concentrations resulted in significant increases in CD54 expression in case of PBQ and formaldehyde. Cell viability was not affected significantly except at the top concentration of PBQ (9 mM). Treatment with non sensitizing chemicals (Fig. 5 b) did not significantly affect detectable levels of CD54 in the absence of cytotoxicity. SDS (0.3 and 3 mM) reduced cell viability below 50% and caused a related change in ΔMFI.

Discussion

The working assumption was that the association of haptens with membrane proteins would inhibit the recognition of epitopes by complementary specific antibodies. To test this hypothesis, attention focused initially on DNCB and its ability to inhibit the detection of specific membrane proteins expressed by THP-1 and U-937 cells. The results demonstrate that treatment of cells with DNCB resulted in a reduced level of detectable membrane proteins (CD14 and CD54). Inter-experimental variations were substantial if the levels of protein expression were relatively low (e.g. CD14 on both cell lines), or if cells had been activated with PMA (THP-1 and CD54). The most reproducible and reliable results were obtained using U-937 cells in combination with CD54 as the membrane determinant. The results obtained are consistent with data reported by Hirota and co-workers in which reduced levels of free cysteine and lysine residues on the cell surface were found following treatment with DNCB (Hirota *et al.* 2009).

We wished to determine whether the reduced detection of CD54 on U-937 cells was due to blocking antibody recognition by DNCB, or due to induced down-regulation of expression of CD54 by internalization. To this end we examined whether inhibition of metabolic activity of U-937 cells influenced DNCB-mediated changes in detection of CD54. Reversible inhibition of metabolic activity by cooling on ice was found to reduce the ability of DNCB to inhibit antibody-mediated detection. In addition, irreversible inhibition using formaldehyde or sodium azide reduced significantly or completely the effects of DNCB. Collectively, these observations suggest that the reduced ability to detect CD54 following treatment of U-937 cells is, at least in part, attributable to an active down-regulation of the membrane protein induced by exposure to DNCB. In line with this is the fact that the DNP hapten was not detectable on cell surface molecules, but intracellular proteins haptenated with DNP were found following exposure of untreated cells or cells treated with sodium azide to DNCB. These data are also consistent with active down-regulation and internalization of CD54.

Although the molecular interactions are very different, it is proposed that the covalent binding of DNCB or the binding of antibody to membrane proteins can cause comparable internalization of CD54 (Lane *et al.* 1991). We demonstrate that treatment of U-937 cells with an anti-CD54 antibody caused a down-regulation of the membrane determinant by internalization, and that this modulation was generally impaired if cells were cooled on ice or treated with sodium azide. Thus antibody modulation displays similar characteristics to the impact of treatment with DNCB on cell surface markers. Unlike antibodies, which are able to cross-link proteins due to their bivalent nature, DNCB has only a single leaving group and thus will not be able to cross-link membrane proteins by crosslinking on a single molecule basis. It may be that the ability, in theory at least, of DNCB to interact with multiple proteins, and to confer more than one DNP molecule to individual target proteins, provides an alternative to cross-linking as a signal for internalization.

Treatment of viable U-937 cells with other haptens (PPD, oxazolone, PBQ, and formaldehyde) resulted in variable effects, however, non-sensitizing skin irritants were clearly without effect. Although DNCB and PPD displayed a clear ability to inhibit antibody-mediated detection of CD54, the other haptens did not. In some instances an increase of the expression of CD54 was observed. This apparent paradox is likely attributable to the fact that contact allergens are able to stimulate an increase in the membrane expression of CD54 in other cell systems. For example, it has been demonstrated that treatment of THP-1 cells with skin sensitizing chemicals like DNCB, PPD, PBQ, formaldehyde and oxazolone resulted in increased CD54 levels to relative fluorescence intensity of 140 or higher, but not if treated with salicylic acid and SDS (Ashikaga *et al.* 2006; Nukada *et al.* 2012). Therefore the two processes may take place in parallel. That is, haptens may stimulate both inhibition of detection of certain membrane proteins by specific antibodies, possibly via active internalization, and also the increased expression of those same proteins. Thus the amount of membrane marker detected will be dependent upon the balance between these processes.

The approach explored here was predicated on the assumption that skin sensitizing chemicals will have the ability to form stable associations not only with soluble proteins, but also proteins expressed at the plasma membrane of cells. The working hypothesis was that the association of a chemical allergen with a specific membrane protein would compromise recognition by an antibody against this protein. Although it is not possible to exclude steric hindrance as a possible contributor to reduced detection of membrane proteins following exposure of cells to DNCB, the data presented here suggest that conjugation of DNCB with cell surface protein does indeed result in a reduced expression which is associated with internalization of the target protein. This may provide another, and possibly more important, mechanism for the trigger of skin sensitization, with internalization of haptenated protein possibly providing the stimulus for changes in intracellular signaling and hence cell activation status. Consistent with this is the finding by Hirota and co-workers (Hirota *et al.* 2009) that treatment with allergen reduced THP-1 cell surface free lysine and cysteine which correlated with phosphorylation of p38 mitogen-activated protein kinase (Hirota *et al.* 2009).

Irrespective of the processes through which antibody detection of membrane protein was reduced, the evidence is that the approach is able to detect the reference contact allergen DNCB and one other sensitizing chemical (PPD). With other contact allergens the results were variable and less persuasive, possibly because there is a dynamic equilibrium between reduced detection and elevated expression of membrane CD54. Consequently, it is premature to suggest that this particular approach shows promise. However, neither is it appropriate to conclude that the strategy is without merit. It is possible that the use of other markers that are known not to be up-regulated on cells by contact allergens may provide a more robust platform and could provide a supplement in test strategies for skin sensitization as published previously by Bauch and co-workers (Bauch *et al.* 2012).

Conflicts of interests

Caroline Bauch, Bennard van Ravenzwaay and Robert Landsiedel are employees of BASF SE, a chemical company intending to use in vitro assays to test their chemicals and products. These authors are, however, not aware of any conflict of interest.

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Fig. 1 Membrane marker expression profiles of resting, PMA-activated and allergen-treated U-937 and THP-1 cells.
U-937 (panel a) and THP-1 (panel b) cells were cultured in medium (resting) (i, ii, iii) or in the presence of PMA for 24 h or 72 h (iv, v, vi). Membrane expression of the markers CD86, CD54 and CD14 was assessed by flow cytometric analysis of 10^4 cells. Representative histograms showing the pattern of expression of each of the membrane markers (open) versus mouse IgG1 κ isotype control antibody (black) are displayed (CD86: i, iv; CD54: ii, v; CD14: iii, vi). The horizontal line depicts the gate set for positive expression for each marker, with the percentage positive cells recorded for each example. (Panel c) Resting U-937 (i), PMA-activated U-937 (ii) or PMA-activated THP-1 cells (iii) were incubated with medium alone (open), DMSO vehicle control (dashed) or with 5 mM DNCB (grey) for 2 h at RT. Membrane expression of CD54 was assessed by flow cytometric analysis of 10^4 cells: representative histograms from one independent experiment showing the pattern of expression following each treatment versus mouse IgG1 κ isotype control antibody (black) are displayed.

Fig. 2 Changes in membrane marker expression profiles provoked by DNCB
Resting viable (■) or formaldehyde-fixed (▲), PMA-activated viable (□) or formaldehyde fixed (Δ) U-937 (ai, iii; bi) or THP-1 (aii, iv; bii) cells were incubated in medium alone (control cells), or in the presence of DMSO or various concentrations of DNCB for 2 h. In some experiments, resting (♦) or PMA activated (◊) cells were incubated for 24 h. Cell viability was measured by PI exclusion (ai, aii) and is displayed as percentage of viable cells for resting (■) or PMA-activated (□) cells after 2 h incubation or for resting (♦) or PMA activated (◊) cells after 24 h incubation. Membrane expression of CD54 (aiii, aiv) and CD14 (bi, bii) was assessed by flow cytometric analysis of 10^4 cells per treatment group and is displayed as delta mean fluorescence intensity (ΔMFI) values compared with concurrent medium control cells (defined as 0 ΔMFI). Results are displayed as mean ± SE of n=3 independent experiments. Statistical significance of differences between DMSO alone and DNCB-treated groups was assessed by Students' *t*-test; $p<0.05$ = *; $p<0.01$ = **, $p<0.001$ = ***.

Fig. 3 DNCB-induced changes in membrane marker expression: role of uptake and modulation
Resting U-937 cells were incubated in medium alone (control cells), or in the presence of DMSO alone or various concentrations of DNCB for 2 h at RT (i), on ice (ii), or in the presence of 20mM sodium azide (iii). Membrane expression of CD54 (panel a) was assessed by flow cytometric analyses of 10^4 cells per treatment group and is displayed as ΔMFI values compared with concurrent control cells (defined as 0 ΔMFI). For detection of intracellular bound DNP, cells were permeabilised with 0.3% saponin, stained with anti-DNP antibody and AlexaFluor labelled secondary antibody. Data are displayed as the fold change (panel b) in anti-DNP antibody binding (MFI values) relative to the background levels recorded in medium control treated cells (defined as 1). Results are displayed as mean ± SE of n=3 independent experiments. Statistical significance of

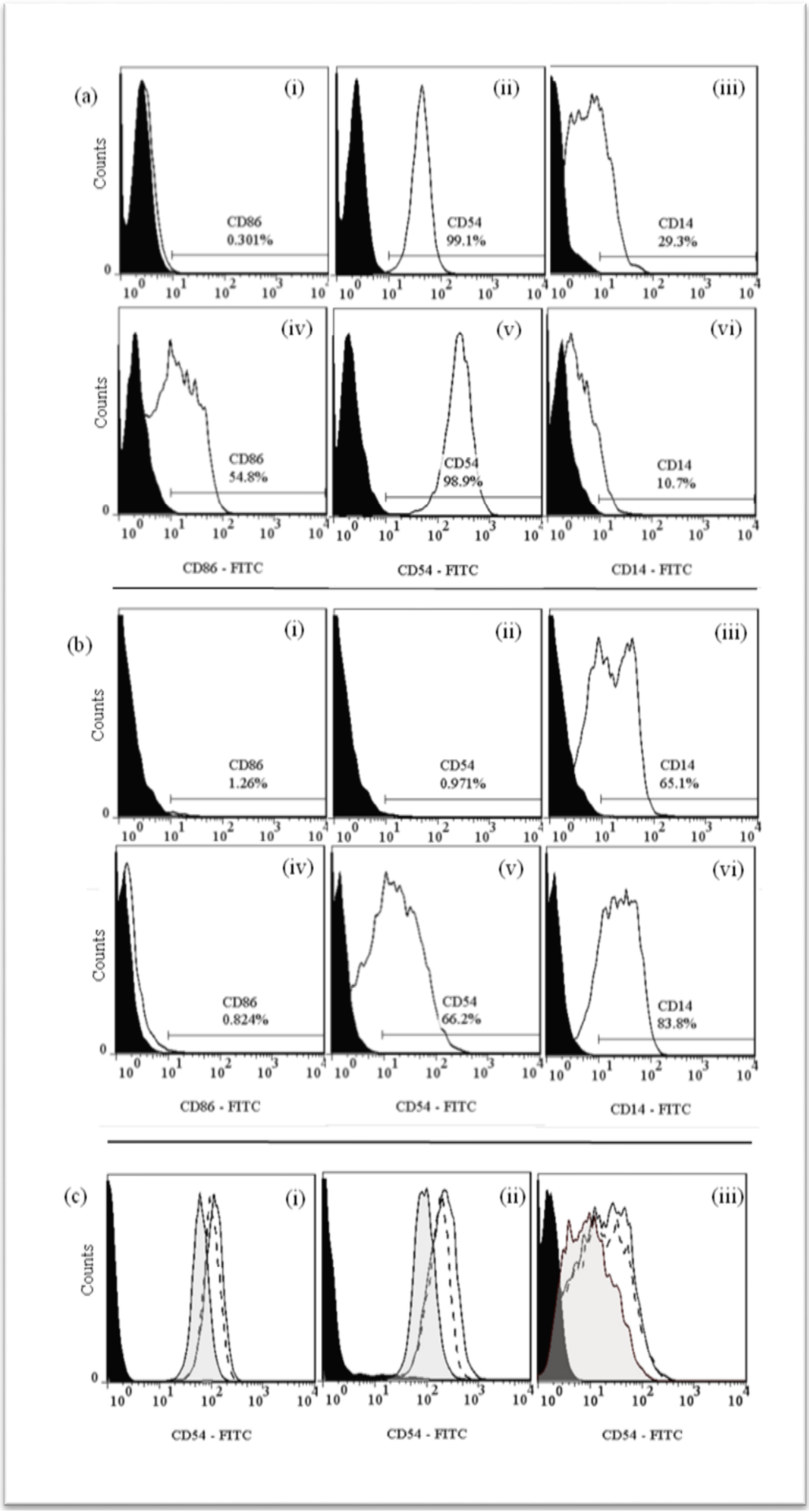
differences between DMSO alone and DNCB-treated groups was assessed by t-test; $p<0.05$ = *; $p<0.001$ = ***.

Fig. 4 Modulation of CD54 protein uptake

Resting U-937 cells were incubated in the presence of anti-CD54 antibody for 0.5 to 2 h at RT (i), on ice (ii), or in the presence of 20mM sodium azide (iii). Cells were washed and stained with FITC labelled secondary antibody. For the purposes of calculating ΔMFI for all treatment groups, control cells were incubated with antibody for 0.5 h on ice (defined as 0 ΔMFI). Results are displayed as mean ± SE of n=3 independent experiments. Statistical significance of differences between DMSO alone and DNCB-treated groups was assessed by t-test; $p<0.05$ = *; $p<0.01$ = **, $p<0.001$ = ***.

Fig. 5 Changes in CD54 expression profiles provoked by other allergens and irritants

Resting U-937 cells were incubated in medium alone (control cells), or in the presence of DMSO or increasing concentrations of allergens (panel a: i,ii para-phenylenediamine; iii, iv oxazolone; v, vi para-benzoquinone; vii, viii formaldehyde) or irritants (panel b: i, ii salicylic acid; iii, iv sodium dodecyl sulfate) for 2 h at RT. Cell viability was measured by PI exclusion (panels a and b: ii, iv, vi, viii) and is displayed as percentage of viable cells. Membrane expression of CD54 (panels a and b: i, iii, v, vii) was assessed by flow cytometric analysis of 10^4 cells per treatment group and are displayed as ΔMFI values compared with concurrent control cells (defined as 0 ΔMFI; horizontal line). Results are displayed as mean ± SE of n=3 independent experiments. Statistical significance of differences between DMSO alone and chemical-treated groups was assessed by t-test; $p<0.05$ = *; $p<0.01$ = **, $p<0.001$ = ***.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(b)
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Fig. 1

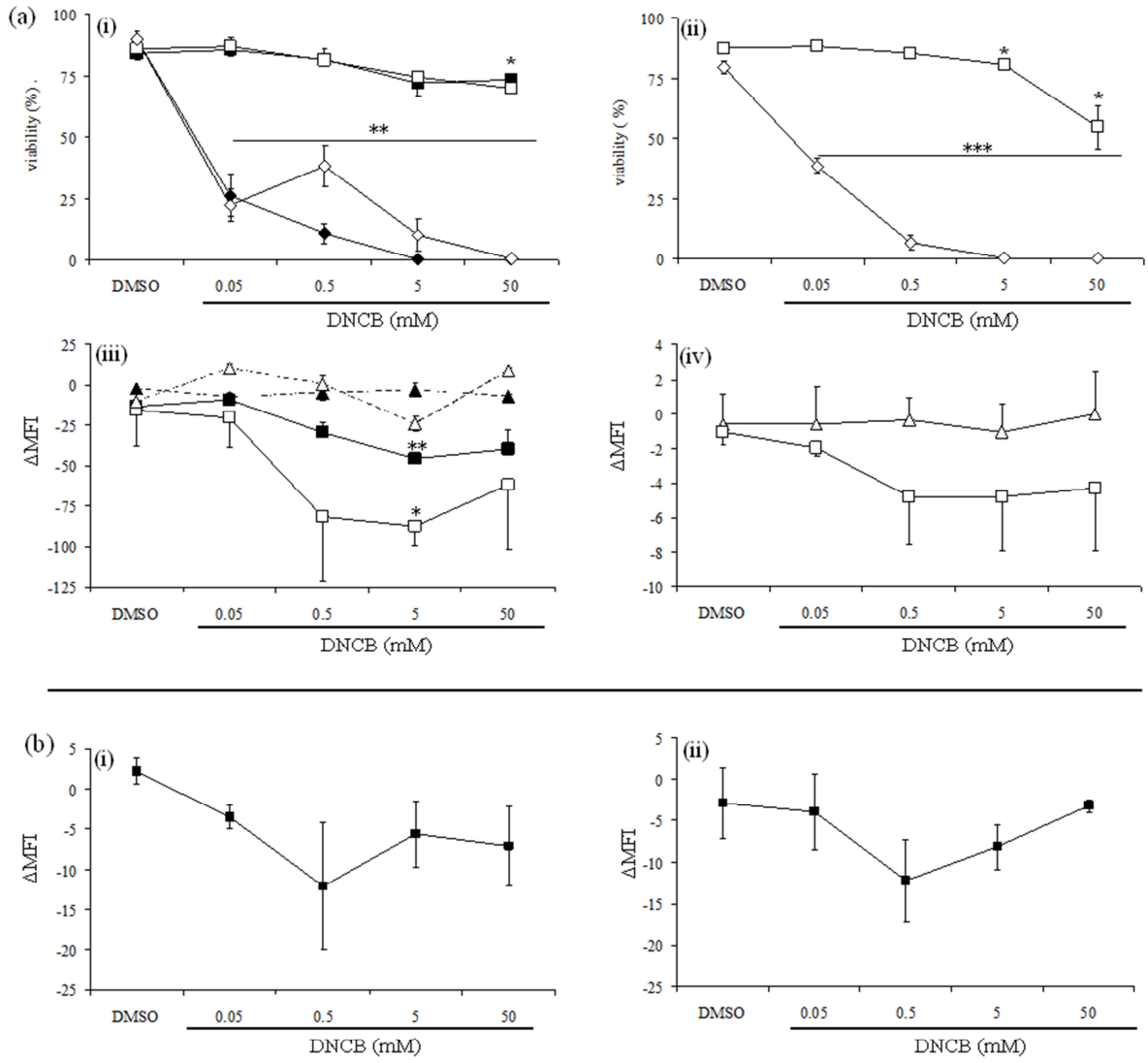

(a)
(i)
viability (%)
DMSO
0.05
0.5
5
50
DNCB (mM)
(ii)
viability (%)
(iii)
ΔMFI
(iv)
(b)
(i)
(ii)

Fig. 2

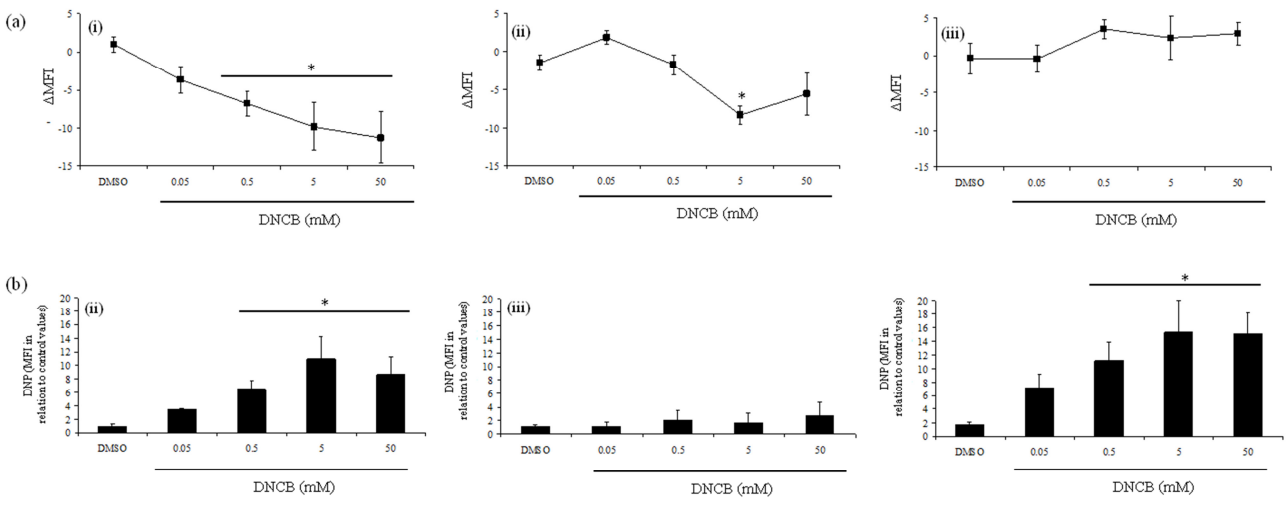

(a)
(i)
(ii)
(iii)
ΔMFI
DMSO
0.05
0.5
5
50
DNCB (mM)
(b)
DNP (MFI in relation to control values)

Fig.3

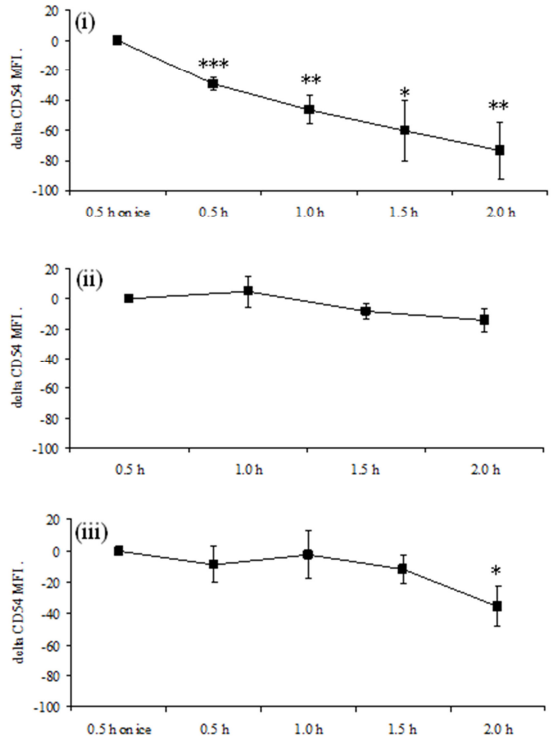
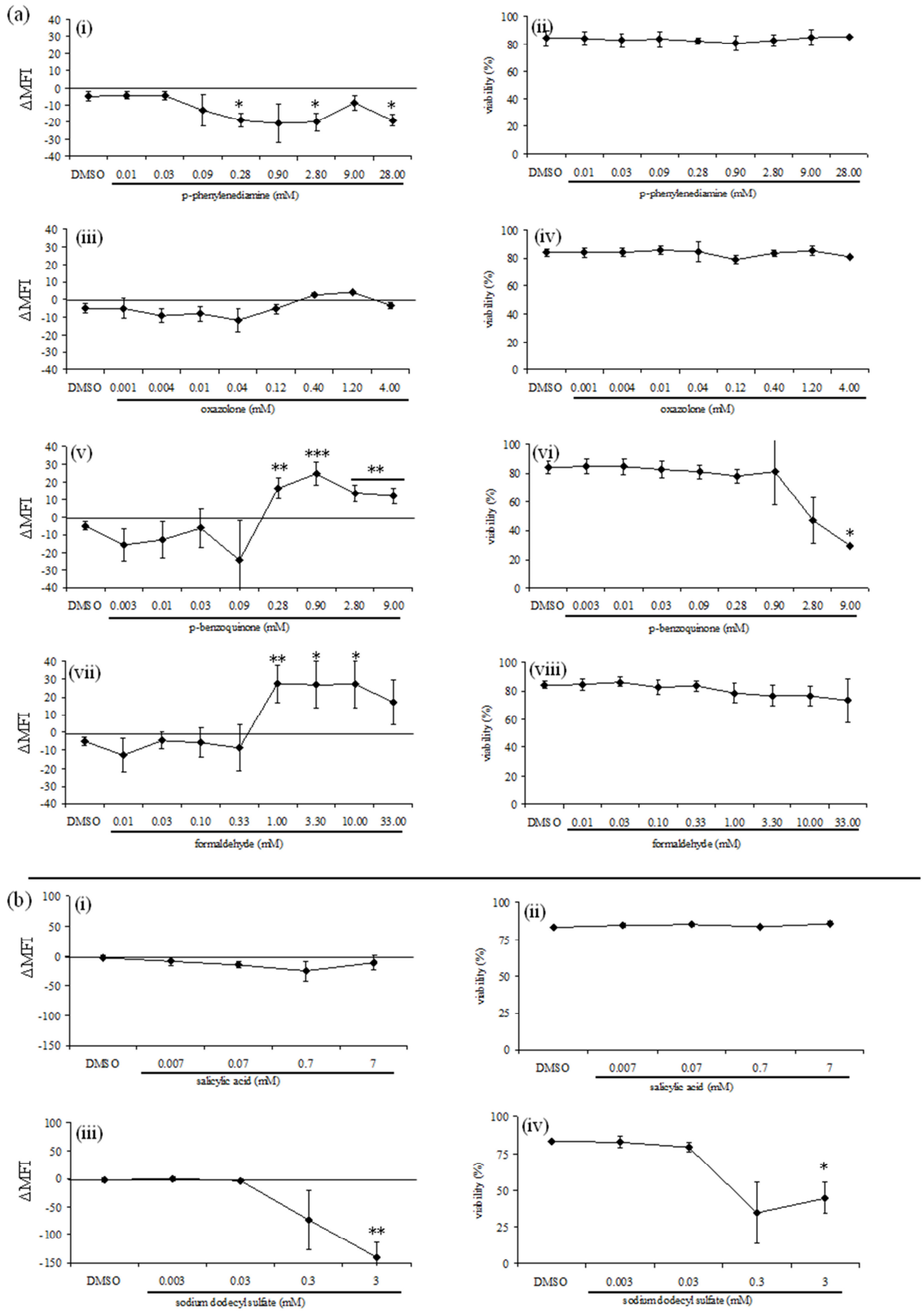

(i)
delta CD54 MFI .
20
0
-20
-40
-60
-80
-100
***
**
*
**
0.5 h on ice
0.5 h
1.0 h
1.5 h
2.0 h
(ii)
delta CD54 MFI .
0.5 h
1.0 h
1.5 h
2.0 h
(iii)
delta CD54 MFI .
*
0.5 h on ice
0.5 h
1.0 h
1.5 h
2.0 h

Fig. 4


(a)
(i)
ΔMFI
DMSO 0.01 0.03 0.09 0.28 0.90 2.80 9.00 28.00
p-phenylenediamine (mM)
(ii)
viability (%)
DMSO 0.01 0.03 0.09 0.28 0.90 2.80 9.00 28.00
p-phenylenediamine (mM)
(iii)
ΔMFI
DMSO 0.001 0.004 0.01 0.04 0.12 0.40 1.20 4.00
oxazolone (mM)
(iv)
viability (%)
DMSO 0.001 0.004 0.01 0.04 0.12 0.40 1.20 4.00
oxazolone (mM)
(v)
ΔMFI
DMSO 0.003 0.01 0.03 0.09 0.28 0.90 2.80 9.00
p-benzoquinone (mM)
(vi)
viability (%)
DMSO 0.003 0.01 0.03 0.09 0.28 0.90 2.80 9.00
p-benzoquinone (mM)
(vii)
ΔMFI
DMSO 0.01 0.03 0.10 0.33 1.00 3.30 10.00 33.00
formaldehyde (mM)
(viii)
viability (%)
DMSO 0.01 0.03 0.10 0.33 1.00 3.30 10.00 33.00
formaldehyde (mM)
(b)
(i)
ΔMFI
DMSO 0.007 0.07 0.7 7
salicylic acid (mM)
(ii)
viability (%)
DMSO 0.007 0.07 0.7 7
salicylic acid (mM)
(iii)
ΔMFI
DMSO 0.003 0.03 0.3 3
sodium dodecyl sulfate (mM)
(iv)
viability (%)
DMSO 0.003 0.03 0.3 3
sodium dodecyl sulfate (mM)

Fig, 5

7.2 ADDITIONAL WORK ON HAPTENIZATION IN VITRO

On the basis of work shown in chapter 7.1 the hypothesis was made that haptenization of proteins and chemical sensitizer lead to reduced antibody binding but also to internalization of the protein on the cell surface. Additional work was performed to prove whether antibody affinity to protein is reduced after its conjugation with chemicals. The possible mechanisms are proposed and are shown in Fig. 5 in chapter 7.1. Protein conjugates were prepared with model proteins and the known chemical sensitizer DNCB and 1-fluoro-2,4-dinitrobenzene (DNFB).

Analysis of protein conjugates with an inhibition enzyme linked immunosorbent assay indicated that conjugation of proteins with DNFB displaced binding antibody. Conjugates prepared with DNCB required DMSO as solvent and did not show any effect on antibody binding. Changes of conditions during conjugate preparation did not results in qualitative conjugates.

Nevertheless this approach showed that the chemical sensitizer DNFB forms stable conjugates with proteins and that this conjugation can be detected using antibody directed against the protein. This method was not applicable for DNCB and it was presumed that the presence of DMSO somehow interferes with the reaction conditions required to form stable conjugates. Nevertheless, the results indicate that chemical conjugation in general can interfere with antibody binding. With regards to the results shown in chapter 7.1 it is indicated that chemical conjugation with cell surface protein may have an effect on the detection using antibody.

The manuscript for this work is currently under preparation.

8 DISCUSSION

The purpose of this work was the development, establishment and validation of animal-free test methods to address different steps of the AOP of skin sensitization induced by chemicals and to propose a test strategy with selected assays to predict the skin sensitizing potential.

Therefore cell based assays but also *in chemico* and *in silico* based methods were established and validated with a defined set of substances of known sensitizing potential in humans (See chapter 4, 5 and 6) (Bauch *et al.* 2011; Bauch *et al.* 2012; Natsch *et al.* 2011). This first part revealed high predictivities of those methods addressing early events of the AOP, i.e. the haptenization. Therefore the second part of this work focused on this event and investigated interactions of chemical sensitizers and proteins, using surface proteins of DC like cell lines as a model. The aim was to show whether cell surface proteins are the target of chemical sensitizers and if so, if this could be detected using a flow cytometry based analysis and the loss or reduction of antibody binding to the altered protein (Chapter 7) (Bauch *submitted*).

The validation studies of the several assays indicated high accuracies for each single assays (>76%). As expected due to the complex biological process of skin sensitization, none of the shown assay exceeded the predictive capacity of the LLNA (90 % compared to human data). Combination of assays which reflect three different and important steps of the sensitization process according to the AOP, namely protein reactivity (DPRA), KC response and indirect protein reactivity (LuSens and/or KeratinoSens) and DC activation (MUSST) reached remarkable predictivity of 94 % and thereby exceeded the LLNA for the given selection of chemicals. In addition the combination of three assays offered higher accuracies compared to other published ITS, although it needs to be considered that the selection of chemicals differed in the number of chemicals and also the composition. McKim and co-workers discussed a combination of a glutathione peptide reactivity assay with gene expression analysis for Keap1/Nrf2/ARE/EpRE, ARNT/AhR/XRE or Nrf1/MTF/MRE pathways regulated genes and showed for 67 tested substances an accuracy of 84 % (McKim *et al.* 2010), whereas Natsch and co-workers described an ITS with peptide reactivity, induction of ARE-dependent luciferase activity in a cell-based assay, *in silico* prediction using TIMES-SS and calculated octanol–water partition coefficient. This ITS reached an accuracy of 86 % with a total number of 116 tested substances (Natsch *et al.* 2009). The ITS described in Chapter 6 and shown in Figure 4 offers some advantages. Besides the prediction of the absence or presence of a skin sensitization potential, one-sided question can be of higher relevance (e.g. can we exclude a skin sensitization potential?). A testing battery offers the possibility to specifically address those questions: Combining the peptide reactivity analysis with the KC reporter gene assay gives results were the sensitizing potential can be excluded with high probability. Combination of both assays yielded in a high sensitivity (100 %). If in addition an assay or combination is selected with a high specificity, the prediction of sensitizing potential can be performed with high probability. Within the tested substances the MUSST showed a specificity of 100 % and had thus a predictive value for positive results of 100 %. Thus being able to predict or vice versa to exclude the sensitizing potentials of chemicals this strategy could be useful for screenings during the early process of development of new compounds.

Reactions of chemical sensitizers with proteins in the skin constitute one important step in early process of skin sensitization Although the necessity of the conjugate formation is known, very little is known about the target proteins, which react with the haptens, and their fate. Sensitizers react with amino- and thiol moieties of the respective amino acids - like lysine and cysteine – in a protein. Thus any lysine or cysteine containing protein may be targets of the chemical reaction. It was discussed that chemical reaction formally reflects post translational modification and leads to changes in function, conformation and protein-protein interaction (Martin 2012; Todd *et al.* 2010; Walter and Ron 2011). This was described to induce the activation of unfolded protein response and following endoplasmatic reticulum stress, such as disrupted protein folding (Todd *et al.* 2010; Walter and Ron 2011). In the work presented here the analysis of cell surface proteins after treatment with sensitizers (see chapter 7) indicated a reduced levels of cell surface proteins after treatment with sensitizers. Further the results indicated that reduced detectable levels might be a combination of inhibited antibody binding and uptake of the changed cell surface determinant. These results correlate with the study of Hirota and co-workers. They reported reduced levels of free cysteine and lysine residues on the cell surface and a subsequent activation of DC after chemical treatment, whereas treatment with already conjugated chemicals did not affect the level of free amino acids on the cell surface and failed to trigger DC activation (Hirota *et al.* 2009). Again this may indicate that the conjugation of proteins on the cell surface of DC and subsequent internalization is an important trigger to activate DC.

The development of alternatives to animal experiments is challenging as complex processes, which may affect each other, have to be simulated *in vitro*. Moreover several technical hurdles had to be overcome during the validation studies and the development of the cell based method. The concentrations of solvents like DMSO, especially in DC activation assays, have to be limited to avoid solvent specific effects or cell activation. Thus the solvent concentration could not be increased to dissolve substances with low solubility. Three test substances had to be excluded from the testing in the DC activation tests due to precipitation, and two more due to technical reasons and interference with the measuring device. Formation of DNCB conjugates failed presumed with the presence of DMSO and probably interferences with the reaction conditions required to form stable conjugates. So far no other vehicles like ethanol, acetone or acetonitrile have been tested on these test systems to omit use of DMSO in such cases. Nevertheless, most solvents trigger activation of cells if applied at higher concentrations. Alternatively to the increase of solvent concentration the substance could be applied as a saturated solution or by passive dosing using a silicon ring saturated with the test substance and applied to the cell culture. Thus the use of solvent during the treatment can be avoided (Kramer *et al*. 2010; Smith *et al.* 2010a; Smith *et al*. 2010b; Tanneberger *et al*. 2010).

The effect of the test substances on cell viability (CV) is of great importance. Cytotoxicity can cause non-specific effects and artifacts and the neglect of cytotoxic effects may yield false positive results. As an example, the Keap1-Nrf2-ARE signaling pathway is responsive to oxidative stress, e.g. due to the appearance of ROS, as well as to electrophilic chemicals. Thus, non-sensitizers may activate an alternate pathway, also known as rescue pathway for cells (Dinkova-Kostova *et al.* 2005; Wakabayashi *et al.* 2004; Wang and Jaiswal 2006), as response to stress and the threat of cell death rather than in response to the sensitizing potential of the chemical.

Additionally, some substances showed dose dependent activity in the reporter gene or DC activation assay albeit accompanied by effects on CV. These so-called borderline substances are difficult to interpret and the definition of CV thresholds is obligatory as a quality criterion. Literature describes such threshold for the herein used assays and substance concentrations reducing CV below 70 % (e.g. KeratinoSens, LuSens and MUSST) or 50 % (h-CLAT) should be excluded from evaluation to avoid false positive predictions (Ashikaga *et al.* 2006; Natsch *et al.* 2011; Nukada *et al*. 2011; Python *et al*. 2007). In addition to this the choice of an appropriate assay to determine CV is of equal importance and may influence the result. For instance DNA of dead cells can be stained with propidium iodide or 7-aminoactinomycin, both that permeate through the porous membrane of dead cells but not into living ones. Based on this principle the identification the exclusion of dead cells is performed. Assess of metabolic activity, e.g. with substrate the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or widely known as MTT substrate, indicates the relative number of living cells. The MTT substrate is actively converted by enzymes in Mitochondria into its purple formazan salt with a measureable absorbance at 490 nm. Thus the increase of formazan and the related increase of absorbance is in proportion with the number of living cells. Although interferences may occur with colored test substances or reducing properties of the chemicals this assay is widely used for adherent cells (Carmichael *et al.* 1987; Wang *et al.* 2010). Those side effects could be easily excluded by means of preliminary test with cell free samples.

The selection of the cell model is dependent on prerequisites like availability of primary human samples, like peripheral blood, or permission to work with such material. Although primary cells may represent a more reliable model, the use of stable cell lines is for above mentioned reasons more convenient and ensures in addition more stable results. Nevertheless cell lines have some disadvantages such as the lack of metabolic capacity. This needs to be considered since some chemical sensitizers are pro-haptens as described above. Solutions were already described in the literature to address this by adding so-called S9 mix extracted from rat hepatocytes (Chipinda *et al.* 2011) or a skin like cocktail of recombinant enzyme (Bergstrom *et al.* 2007). The benefit of metabolic capacity of KC can be utilized in co-culture with DC (Hennen *et al.* 2011; Schreiner *et al.* 2007). Several markers are available for DC activation like release of cytokines (IL-1α/β or TNFα), a range of cell surface makers and the ability of DC to migrate towards different chemokines chemokines as a result of chemokine receptor expression (Ouwehand *et al.* 2010). Not every cell line is able to express those markers as a result of DC activation and some show a remarkable level of expression even in their immature state (e.g. U-937 cells and CD54 (Huang *et al.* 2001)). The robustness of each assay is correlated with the selected marker, which needs to be shown in validation studies as described in this work (see chapters 4,5,6). To address this, the selection of chemicals used in optimization and validation is important and should reflect a wide range of sensitizers, from weak to extreme as well as prohaptens, non-sensitizers and irritants. Thus weaknesses of the selected marker, cell line or assay can be detected.

9 CONCLUSION

The work for this dissertation investigated on animal-free test methods to assess for skin sensitizing potential of chemicals. ACD and especially the sensitization process triggered by chemical allergens are complex not only due to several, consecutive steps and this is described as well as the AOP of skin sensitization. The presented assays can be directly addressed to different steps of this mentioned AOP. The DPRA and computer based analysis with the OECD OSAR toolbox reflect the protein reactivity and haptenization of chemical sensitizer and proteins. The response of KC and their subsequent activation is mirrored with the reporter gene analysis in the KC cell assay KeratinoSens and LuSens. With the DC cell like activation assays MUSST and h-CLAT is the activation and maturation of DC addressed. The validity, reliability and reproducibility of these assays was shown for more than 50 chemicals and indicated an accuracy greater than 76 % for every assay. The cell based assays (KeratinoSens, LuSens, MUSST and h-CLAT) benefit from the use of cell lines instead of primary cells. Not only the limited availability of primary cells also the donor-to-donor variability is avoided with the use of stable cell lines and thus robust and reliable test systems are provided. Nevertheless changes in sensitivity may occur over the time of cell maintenance and with increasing passage number and records to background signals, constitutive expressed proteins and the use of positive control and , although not essential, negative control substances ensure high quality and reliability of results.

The formation of protein conjugates with chemicals sensitizers is crucial as no cell response and activation of DC can be triggered without, although very little is known about the target protein. With the DPRA and its high predictivities and the investigations on the haptenization of cell surface proteins it can be presumes that not only the haptenization but also the internalization of conjugated protein may be involved in activation of DC. Nevertheless this needs to be further investigated with more test substances and an alternative protein determinant, whose expression is not regulated during the process of maturation of DC.

With one *in vitro* assay the process of skin sensitization is not sufficiently reflected, but with the combinations of those assays into testing strategies a closer simulation of the *in vivo* process is allowed. It was shown that with combination of three assays an increase in predictivities can be achieved. The proposed test strategy which combines peptide binding with KC activation and evaluates DC activation using MUSST yielded in the highest predictivity with an accuracy of 94% and only three false predicted substances. One important feature in the assessment of the skin sensitizing potential is missing in the presented testing strategy: assessment of sensitizing potency. Till now only a binary answer only (sensitizer yes/no) is provided by this *in vitro* test strategy. Nevertheless, this work took some important steps towards the use animal-free methods for hazard identification of skin sensitizers and the replacement of the current existing animal models, although no regulatory acceptance was obtained yet for any of the here mentioned methods.

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Internet link to software OECD QSAR toolbox

http://www.oecd.org/chemicalsafety/assessmentofchemicals/theoecdqsartoolbox.htm)

11 LIST OF FIGURES

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13 APPENDIX

13.1 ABBREVIATIONS

3R	Reduction, Refinement, Replacement
ACD	allergic contact dermatitis
AOP	Adverse outcome pathway
APC	antigen presenting cell
ARE	antioxidant response element
BrdU	5-Brom-2-desoxyuridin
CCR	C-C chemokine receptor
CESAR	Computer assisted Evaluation of industrial chemical Substances According to Regulation
CV	Cell viability
iDC	Immature dendritic cell
DMSO	dimethyl sulfoxide
DNCB	1-chloro-2,4-dinitrobenzene
DPRA	direct peptide reactivity assay
DST	Dermal sensitization threshold
ECHA	european chemical agency
ECVAM	European Centre for Validation of Alternative Methods
EPA	Evironmental Protection Agency
FDA	US Food and Drug Administration
GARD	genomic allergen rapid detection test
GSH	glutathione
GPMT	guinea pig maximization test
h-CLAT	human cell line activation test
HPLC-UV	High pressure liquid chromatography with ultraviolet detection
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICAM-1	Intercellular Adhesion Molecule 1
IL	Interleukin
ITS	Integrated testing strategy
KC	keratinocyte
Keap1	Kelch-like ECH associated protein-1
LC	Langerhans' cells
LC-MS	liquid chromatgraphy – mass spectrometry
LLNA	local lymph node assay
MHC	major histocompatibility complex
MUSST	Myeloid U937 skin sensitization test
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
NC3R	National Centre for the Replacement, Refinement and Reduction of Animals in Research
NCA	Netherlands Centre Alternatives to Animal Use
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
Nrf2	Nuclear factor (erythroid derived 2)like 2
OECD	Organization for economic co-operation and development
QSAR	quantitative structure-activity relationship
REACH	Registration, Evaluation, Authorisation of Chemicals
RHE	reconstructed human epidermis
ROS	reactive oxygen species

SDS	sodium dodecyl sulfate
SI	stimulation indices
SN2/AR	nucleophilic (aromatic) substitutions
TCR	T cell receptor
TNFα	Tumor necrose factor α
TTC	Threshold of Toxicological concern
UV-HPLC	Ultra violet- high pressure liquid chromatography
WoE	Weight of Evidence
ZEBET	*Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch*

13.2 AFFIRMATION

Caroline Dorothee Bauch

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Eppingen, den 05.02.2013

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich meine Dissertation selbstständig und nur mit den angegebenen Hilfsmitteln angerfertigt habe.

Caroline D. Bauch

Caroline Dorothee Bauch Eppingen, den 05.02.2013

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Erklärung

Ich erkläre hiermit, noch keinen Promotionsversuch unternommen zu haben.

Caroline D. Bauch

13.3 SUBMISSION LETTER

Betreff:	Submission Confirmation
Von:	Toxicology in Vitro (tiv@elsevier.com)
An:	caroline.bauch@yahoo.de;
Datum:	11:36 Sonntag, 3.Februar 2013

Toxicology in Vitro
Title: Characterization of haptenization of cell surface proteins by skin sensitizing chemicals
Authors: Caroline Bauch; Rebecca J Dearman; Ian Kimber; Bennard van Ravenzwaay; Robert Landsiedel
Article Type: Research Paper

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Curriculum Vitae

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Date of birth:	30.12.1985
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Research experience

01.06.2011 – 31.08.2012	**PhD Exchange Student** University of Manchester, Faculty of Life Sciences Laboratory of Prof. Ian Kimber and Dr. Rebecca Dearman
16.03.2009 – 31.10.2009	**Diploma student** BASF SE, Experimental Toxicology and Ecology *"Establishment and optimization of dendritic cell activation assay protocols of three human monocytic cell lines for the prediction of skin sensitization" (Grade 1.0)*
01.08.2008 – 31.01.2009	**Diploma Internship** *Forschungszentrum Karlsruhe in der Helmholtz Gemeinschaft* – Karlsruher Institute of Technology, Institute of Functional Interfaces *"Establishment of activity assays for lysozyme and cloning optimized cDNA for the expression of wild-type hen-egg white lysozyme in Pichia pastoris"*
01.02.2007 – 15.03.2007	**Student research project** Ruprecht-Karls-University Heidelberg; Medical Faculty Mannheim, Molecular alcohol research in gastroenterology *"Effect of CYP inhibitors on the toxicity of TGF-β and ethanol in primary mouse hepatocytes" (Grade 1.7)*
01.03.2006 – 26.08.2006	**Diploma Internship** Ruprecht-Karls-University Heidelberg Medical Faculty Mannheim, Molecular alcohol research in gastroenterology *"TGF-β response and oxidative stress produced by ethanol in mouse hepatocytes and HepG2 cells"*

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01.12.2009 – 30.12.2012	**Graduate student, PhD** BASF SE, Experimental Toxicology and Technical University of Darmstadt *"Development of in vitro test systems to predict skin sensitization potential of chemicals"*
01.03.2005 – 31.10.2009	**Graduate Engineer Biological Chemistry** Mannheim University of Applied Science (Hochschule Mannheim) (Grade 1.9)

01.09.2002 – 31.07.2004	***Berufskolleg für Gesundheit und Pflege I + II*** Advanced technical college entrance qualification and Assistant for health care and welfare

Laboratory skills	
Cell biology	Cell culture, maintenance and cryopreservation of: Stable cell lines: adherent cells (HaCaT, HepG2, BALB/C 3T3 fibroblast) and suspension cells (U-937, THP-1, MUTZ-3) Primary cells: human peripheral blood monocytes Immunofluorescence (Flow cytometric application).
Toxicology/Bioscience	Flow cytometric-based analyses Reporter gene transactivation based assays (luminescence) Cytotoxicity assays (LDH, neutral red uptake, MTT assay, propidium iodide) Dendritic cell activation assay
Molecular biology/Biochemistry	Cloning: bacterial vector system. DNA/RNA isolation techniques (bacteria). Standard PCR techniques and sequencing. Immnobiochemistry (Western blot), Protein biochemistry, ELISA Enzyme activity testing (lysozyme)
Microbiology	Culture of Escherichia coli, Micrococcus luteus and Pichia pastoris

Additional skills

Languages

German (fluent, native speaker)

English (fluent)

Spanish (beginner)

Greek (beginner)

Computational skills

MS-Office (advanced)

PubMed (advanced)

GeneCard (beginner)

EndNote (advanced)

Reference Manager (advanced)

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Eppingen, den 05.02.2013

Publications

In preparation	**Bauch C.**; Dearman R.J.; Kimber I., van Ravenzwaay B.; Landsiedel, R.: *Conjugation with protein reactive chemical inhibits anti-protein IgG binding: potential for identification of chemical sensitizers*
submitted	**Bauch C.**; Dearman R.J.; Kimber I., van Ravenzwaay B.; Landsiedel, R.: *Characterization of haptenization of chemical sensitizer on the cell surface Toxicology in vitro*
2012	**Bauch C.**; Kolle S. N.; Ramirez, T.; Eltze, T.; Fabian E.; Mehling, A.; Teubner, W.; van Ravenzwaay, B; Landsiedel, R.: Putting the parts together: Combining in vitro methods to test for skin sensitizing potentials.; Regul Toxicol Pharmacol. 63 (3), p.489-504; DOI: 10.1016/j.yrtph.2012.05.013; 2012
2011	**Bauch C.**; Kolle S. N.; Fabian E.; Pachel C.; Ramirez, T.; Wiench B.; Wruck, C.J.; van Ravenzwaay, B; Landsiedel, R.: *Intralaboratory validation of four in vitro assays for the prediction of the skin sensitizing potential of chemicals;* Toxicol in vitro 25 (6) SI p. 1162-1168; DOI: 10.1016/j.tiv.2011.05.030; 2011
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Poster presentations at Conference

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Lake district, North-England close to Lake Grasmere

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different path or to gain the strength to remove them. Other stones are really rare and precious, although some of them hide their beauty inside and we have to have the patience and the talent to open the stone without breaking it be able to realize the gift we have got. Nevertheless what kind of stones we can compare the people with we have met or we will meet, they are part of our path and will help us to find and choose the right one. Sometimes it is worth it to look back to realize, that the changes and decisions that have been made were right and that sometimes it just takes a while to realize it.

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